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Report of the Follow-Up Collaborative Study

Determination of the sum of Fumonisin B_1 and B_2 in Compound Animal Feed and Maize by Immunoaffinity Column Clean-up and High Performance Liquid Chromatography with Fluorometric Detection

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1. INTRODUCTION

The accurate determination of mycotoxins in food and feed matrices for which EU legislative limits apply require robust and reliable analytical techniques. The robustness and reliability are best shown through validation by a collaborative study.

Previous collaborative studies dealing with other mycotoxins [1-4] have shown that it is possible to achieve performance characteristics which are fit-for-purpose provided suitable methodology is available. As with any interlaboratory comparison homogeneity between the test units is of utmost importance. Due to the complexity of food and feed matrices particular care has to be taken during test material preparation to achieve this.

Methods for the determination of Fumonisin B_1 (FB_1) and Fumonisin B_2 (FB_2) have been subject to a collaborative study [5] in the past and the methodology used involved immunoaffinity clean-up to purify the sample extracts. Detection was afforded by derivatisation of the Fumonisins to yield fluorescent derivatives before a chromatographic separation. The reagent used was o-phtaldialdehyde and mercaptoethanol.

However, pre-column derivatisation does have disadvantages related to more demanding chromatography and the instability of the derivatives. Strict time control of all processes is required to obtain adequate repeatability which necessitates the use of programmable auto liquid samplers (ALS). This may be circumvented by using post column derivatisation instead.

Here the native Fumonisins are separated and reagents are added constantly to the effluent of the chromatographic column. An additional pump, a mixing Tee, and additional tubing are needed for post column derivatisation replacing the need for a sophisticated ALS. During method development it could be shown that both methods can perform equally well with respect to the requirements by EU legislation for method performance and working range [6].

A collaborative study to validate a method for the "Determination of Fumonisin B_1 and B_2 in Baby Food, Breakfast Cereals and Animal Feed by Immunoaffinity Column Clean-up with High Performance Liquid Chromatography and Fluorometric Detection" [7] failed partially because of problems with the immunoaffinity columns (IAC) used for the study. After modifications to the method protocol regarding a check of proper performance of the IAC and the sample extract clean-up we describe below the results of a repeat of the study for compound animal feed and maize.

2. METHOD OF ANALYSIS

In brief, the method under investigation involved the following: 20 g of test material were extracted with 200 ml of 50 % methanol in phosphate-buffered saline for 2 h. After settling of the particulate matter 5 ml of the supernatant were diluted to 50 ml with phosphate-buffered saline. The diluted extract was filtered and 25 ml of it were applied to an immunoaffinity column. Following a washing step with 10 ml of phosphate-buffered saline the bound analytes were eluted with 5x500 µl methanol followed by 2 ml of water. The eluate was collected and made up to 5 ml

with water. This solution was then injected into a HPLC system. After either pre- or post-column derivatisation with o-phtaldialdehyde and mercaptoethanol or N-acetyl-cystein the derivatised analytes were quantified by fluorometry.

The detailed method protocol can be found in Annex C.

3. LAYOUT OF THE COLLABORATIVE STUDY

This collaborative study was planned according to guidelines of the AOAC Official Methods Program [8]. In particular, this means that five different materials would be measured as blind duplicates covering the concentration range of interest [9] as much as possible. These five materials consisted of three individual materials (Levels B, C, SH) of which two (B, C) additionally had to be spiked by the participants out of two solutions of blinded concentration. The spiked materials were to be used to determine recovery.

17 laboratories of 12 European Union Member States, all of them participants in the initial collaborative study [7], were invited to participate in this follow-up (see Annex A for details). Each laboratory received:

- Six test units identified by a code (blind duplicates of three materials)
- Two test units identified as "Spike A"
- Two test units identified as "Spike B"
- One amber ampoule identified as "Spike solution A"
- One amber ampoule identified as "Spike solution B"
- One test unit identified as "Animal feed blank" (to be used in connection with one of the supplied IAC to optimise the LC separation conditions)
- 14 immunoaffinity columns (IAC)
- Vial with Fumonisin Mix reference material (if it was needed since the volume of the reference material provided for the previous study was insufficient)
- Documentation: Method protocol, Spiking protocol, Cover letter, Copy of Certificate of Fumonisin Mix reference material (if it was requested), Results form, Questionnaire (all these documents can be found in Annex C).

The shipments to the participants were dispatched on 30 Sep 2009 and the deadline for reporting was set to 01 Dec 2009.

4. PREPARATION OF TEST MATERIALS

Various kinds of compound animal feed (rabbit, horse, pig feed) were purchased at local wholesalers. After comminution of the materials with a Romer RAS® mill they were checked for the presence of FB_1 and/or FB_2 and found to be essentially free of the analytes (<140 µg/kg FB_1, <60 µg/kg FB_2).

The comminuted and Fumonisin-free materials were mixed extensively (see Table 1 Blank) in a rotating-drum mixer and then milled with a Retsch centrifugal mill (Model ZM 200) with a 3 mm sieve. This process of mixing and milling was repeated twice with consecutively smaller sieves of 1 and 0.5 mm. A comminuted, highly contaminated maize material was then blended with this homogenous blend of various Fumonisin-free compound animal feeds for the levels B and C or used pure for level SH.

The dilution of the highly contaminated maize material with the blank for Levels B and C was done according to the following scheme: Equal amounts of contaminated and blank material were manually mixed and then milled (Model ZM 200) with a 2 mm sieve. The resulting contaminated material was then again mixed with an equal amount of blank material. This was repeated until sufficient amounts of contaminated material at the desired contamination level were obtained. Then the material was divided into 10 subsamples with a sample divider. Each subsample was again divided into 10 new subsamples. This was repeated twice. All subsamples were then combined into two batches following a particular scheme. The two batches were combined and milled again (Model ZM 200) with a 1 mm sieve for the final homogenous material.

Table 1: Composition of test materials

Test Material	Ingredient	Parts	Principal components
Blank	Rabbit feed	4	cereals, seeds, crop by-products, vegetables, minerals
	Horse feed	5	oat, barley flakes, flour pellets, corn flakes, pea flakes, fibres, oil, molasses
	Pig feed	5	peas, roasted soy, wheat, barley, tapioca, cabbage seeds, animal grease, corn, salt
Level B	Blank	4	See "Blank"
	Maize	1	Maize
Level C	Blank	2	See "Blank"
	Maize	1	Maize
Level SH	Maize	1	Maize

5. STATISTICAL ANALYSIS

To avoid the need to exclude laboratories with "outlying" results robust statistical methods were used for the estimation of repeatability and reproducibility as described in ISO 5725 Part 5 [10]. In particular, "Algorithm S" ([10], p. 36) was used to obtain a robust estimate of the standard deviation s^* of the differences between the blind duplicates per material and "Algorithm A" ([10], p. 35) to obtain a robust estimate of the standard deviation s_d of the averages of the blind duplicates per material.

The repeatability standard deviation s_r can then be calculated as:

$$s_r = s^* / \sqrt{2} \qquad (1)$$

The between-laboratory standard deviation s_L is derived from s_r and s_d:

$$s_L = \sqrt{s_d^2 - (s_r^2 / 2)} \qquad (2)$$

If the expression under the square root is negative s_L will become zero.

Knowing s_L and s_r the reproducibility standard deviation s_R is calculated as:

$$s_R = \sqrt{s_L^2 + s_r^2} \qquad (3)$$

Relative standard deviations (RSD) were calculated as standard deviation times 100 divided by the mean value:

$$RSD = \frac{100s}{\bar{x}} \qquad (4)$$

Repeatability and reproducibility limits were calculated by multiplying the respective standard deviation with 2.8 which gives roughly a 95 % confidence at two replications:

$$r = 2 * \sqrt{2} * s_r = 2.8 s_r \qquad (5)$$

$$R = 2 * \sqrt{2} * s_R = 2.8 s_R \qquad (6)$$

Student's t-test was used to determine significances of differences between means.

6. IN-HOUSE METHOD PERFORMANCE AND VERIFICATION OF TEST MATERIAL HOMOGENEITY

The method under investigation was validated as recommended ([8], Sec 1.3); in particular, useable calibration range, matrix interferences, recovery, chromatographic performance specifications, and precision were determined.

Since pre- or post-column derivatisation was permissible for this study we determined the useable calibration range for both. With the instrumentation as described in the method protocol (see Annex C) a range from 10 ng/mL to 2000 ng/ml of the individual Fumonisin in the injection solution was usable under both conditions. Significant matrix interferences for different compound animal feeds or maize could not be detected (see chromatograms in Annex C) when observing the stated performance specifications for the HPLC, namely a peak asymmetry factor of $0.9 < As < 1.4$ at 10% of full height, sufficient retention ($k > 2$), and resolution ($R_s > 1$). Apparent recovery was determined to be larger than 80% for both FB_1 and FB_2. Relative repeatability standard deviations ranged from 7 % at ca. 3000 µg/kg to 2 % at ca. 17000 µg/kg for the sum of FB_1 and FB_2. Performance criteria laid down for food analysis Regulation (EC) No.401/2006 [6] were met by this method for feed analysis.

To verify homogeneity of the test materials 10 % of the total number of units per material were picked at random, e.g. 5 for Level B, 5 for Level C, and 10 for Level SH. Two independent determinations were performed per unit with the method under investigation. Sufficient homogeneity was assumed if the between-unit variance (s^2_{sam}) was not significantly different from the within-unit variance (s^2_{an}) with an α-error of 0.05.

The between-unit variance (s^2_{sam}) and the within-unit variance (s^2_{an}) were obtained from one-way analysis of variance (ANOVA). The probability *p* of the two variances being identical was 0.98 for Level B, 0.34 for Level C, and 0.19 for Level SH. Therefore sufficient homogeneity of all three test materials was assumed.

7. RESULTS AND DISCUSSION

Sixteen of the 17 participating laboratories reported back their measurement results for the ten test units they received. One laboratory reported two sets of results. One set was obtained following the method under investigation and the second set was obtained using LC-MS instead of HPLC-FLD.

The laboratories reported individual mass fractions for FB_1 and FB_2 (listed in Annex B). For the purposes of this report we added the individual mass fractions to obtain the sum mass fraction of FB_1 and FB_2 and evaluated only this sum mass fraction (FB_{SUM}). Table 2 lists the calculated sums.

Table 2: Sum mass fractions of FB_1 plus FB_2 in µg/kg for the blind duplicates of the five materials by laboratory. Spike A consists of Level C plus 3287 µg/kg FB_{SUM}, Spike B of Level B plus 2740 µg/kg FB_{SUM}. Grey shading indicates exclusion because of non-compliance.

Lab ID	Spike A		Spike B		Level B		Level C		Level SH	
101	10103	9951	6099	5994	3546	3543	6222	6394	17680	16735
102	8976	8929	5677	5647	3038	3103	5721	5780	15191	15681
105	9571	9340	6140	6132	3426	3526	5044	6698	18576	18179
107	7207	6635	4772	4785	2706	2577	5099	4983	13785	14353
110	7646	7482	4734	4781	2874	2802	5257	4631	14660	14646
112	7888	7719	4767	4680	2716	2765	5118	4726	11791	12300
212	7350	7848	5113	4837	2942	2921	5266	5093	13540	13480
301	8375	9419	4813	4581	4622	4238	7462	9257	23808	20104
302	7874	7869	4853	4795	2868	2828	5482	5409	14960	14728
303	6595	5919	3547	3638	2125	2375	4636	4617	12960	12416
304	8583	8630	4668	4859	3381	3497	6030	5974	19106	19658
305	12267	11077	6149	5875	3886	3350	8210	5256	18524	18973
306	4352	4285	2503	2390	1177	1142	2802	2747	8442	8440
309	979	980	483	508	2503	2675	5153	6113	12033	12577
310	9290	9197	5811	5929	3707	3796	6650	6779	18277	18387

Lab ID	Spike A		Spike B		Level B		Level C		Level SH	
317	8162	7952	4538	4638	2841	2900	5494	5106	16694	16498
410	6828	6683	4141	4089	2480	2447	4584	4378	13578	13550

Five sets of results had to be excluded from the evaluation because of non-compliance (shaded grey in Table 2): Laboratory 102 applied an additive to the mobile phase different from the one explicitly prescribed in the method protocol; Laboratory 305 used LC-MS because of failure of the HPLC-FLD system; Laboratory 306 did not meet the requirements for peak asymmetry (Method protocol clause 3.13.3) for the peaks of FB_1 and FB_2 in the calibration solutions indicating insufficient separation from reagent peaks which might be the cause of consistent underestimation of the mass fractions in the test materials; Laboratory 309 found unexplainable low mass fractions in the two spiked materials; after contacting the laboratory inconsistencies with the calibration (response factors for FB_2 several times larger than for FB_1) were discovered which were in stark contrast to our experience; the additional set of results acquired with LC-MS (see above) is listed with Laboratory ID 410. However, for informative purposes these results are depicted in the respective graphs.

Table 3 lists the performance parameters calculated from the reported values (no correction for individual recovery) as described in Sec. 5 above with exclusion of the non-compliant results.

Table 3: Method performance parameters in ascending order of the mean; Spike B consists of Level B plus 2740 µg/kg FB_{SUM}, Spike A of Level C plus 3287 µg/kg FB_{SUM}; the apparent recoveries were calculated based on these additions; n.a. – not applicable.

Performance parameter	Level B	Spike B	Level C	Spike A	Level SH
Mean value $\bar{x}$ [µg/kg]	3110	5000	5600	8200	16000
Repeatability standard deviation s_r [µg/kg]	81.9	101	295	289	398
Relative repeatability standard deviation RSD_r [%]	2.6	2.0	5.3	3.5	2.5
Repeatability limit r [µg/kg]	229	282	826	809	1120
Reproducibility standard deviation s_R [µg/kg]	589	766	863	1230	3210
Relative reproducibility standard deviation RSD_R [%]	19	15	15	15	20
Horwitz ratio	1.4	1.2	1.3	1.3	1.9

Performance parameter	Level B	Spike B	Level C	Spike A	Level SH
Reproducibility limit *R* [µg/kg]	1650	2140	2420	3450	8980
Apparent recovery [%]	n.a.	69	n.a.	79	n.a.

Based on the Horwitz ratios of 1.2 to 1.9 the performance of the investigated method is adequate. The ratio of 1.9 for the highest level stands out even though RSD_R is comparable to the other levels. The reason is that the predicted RSD_R according to the Horwitz equation is lowest for the highest mass fraction while the primary source of variation, namely the immunoaffinity clean-up step, is the same as for the lower levels. There are no recommendations for performance of methods of feed analysis but performance criteria laid down for food analysis in Regulation (EC) No.401/2006 [6] are met by this method for feed analysis. Only the apparent recovery in Spike B with 69 % is just outside the permissible range of 70 to 110 %.

To show how performance parameters would have changed if a classical approach of evaluation (outlier removal, parametric statistics) had been used Table 4 has been added.

Table 4: Method performance parameters calculated with the classical approach in ascending order of the mean; Spike B consists of Level B plus 2740 µg/kg FB_{SUM}, Spike A of Level C plus 3287 µg/kg FB_{SUM}; the apparent recoveries were calculated based on these additions; n.a. – not applicable.

Performance parameter	Level B	Spike B	Level C	Spike A	Level SH
Outliers removed	0	0	2	0	1
Mean value $\bar{x}$ [µg/kg]	3150	4980	5450	8190	15600
Repeatability standard deviation s_r [µg/kg]	106	96.4	199	310	326
Relative repeatability standard deviation RSD_r [%]	3.4	1.9	3.7	3.8	2.1
Repeatability limit *r* [µg/kg]	298	270	558	868	913
Reproducibility standard deviation s_R [µg/kg]	600	720	691	1110	2510
Relative reproducibility standard deviation RSD_R [%]	19	14	13	14	16
Horwitz ratio	1.4	1.2	1.0	1.2	1.5
Reproducibility limit *R*	1680	2020	1930	3120	7020

Performance parameter	Level B	Spike B	Level C	Spike A	Level SH
[µg/kg]					
Apparent recovery [%]	n.a.	67	n.a.	83	n.a.

It can be said that the classical evaluation approach leads to smaller estimates in most of the cases. This comes at the cost of having to remove outliers which might invite discussions about whether the removal was justified. Using robust statistics avoids this at some what more conservative estimates.

A helpful tool to analyze collaborative study data are graphical displays. One way of displaying such data are so called Youden plots. Youden plots are created when plotting the first result of a duplicate vs. the second for all laboratories. They display in one glance repeatability (closeness of points to the identity line) and reproducibility (tightness of the cloud of points).

Mean & range plots are a second way of displaying the data. Here the mean values and their ranges are plotted in ascending order for all laboratories. Also depicted are the overall mean and the reproducibility limit for two replicates. This kind of plot allows for quick identification of an individual laboratory's bias compared to the overall mean. Figure 1 to Figure 5 shows the two plots (top Youden, bottom Mean & Range) for the sum of the mass fractions of FB_1 and FB_2 for each of the five test materials. The plots for the individual toxins can be found in Annex B.

Of the 16 laboratories which reported back results ten (three non-compliant) used pre-column derivatisation and five post-column. One laboratory (non-compliant) used LC/MS for detection and one of the pre-column laboratories also reported results from LC/MS. Looking at the graphs (Figure 1 to Figure 5) it can be seen that LC/MS delivers results comparable to HPLC-FLD.

It is also evident from the graphs that Laboratory 301 reported results which were the highest for all three naturally contaminated materials (Figures 1, 3, 5). That is a highly unusual finding and suggests a bias not explainable by random variation. Looking at the graphs for the individual fumonisins it can be seen that the main driver behind this seems to be overestimation of FB_2. Furthermore, the differences between the spiked materials and the corresponding naturally contaminated materials were not significantly different from zero. This suggests that the spiking was done improperly. Upon questioning laboratory 301 replied that all procedures were done according to the protocol.

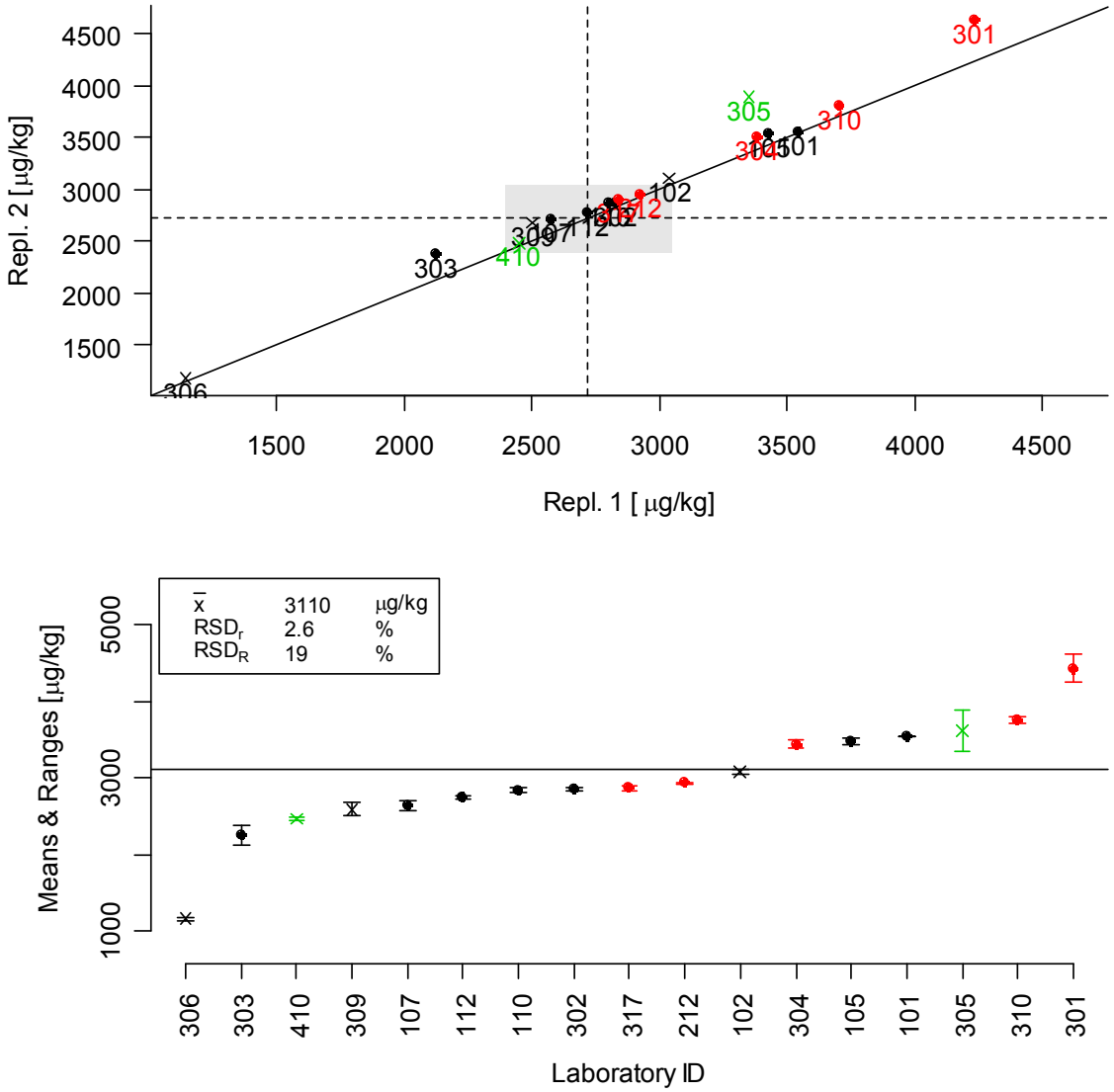

Figure 1, Level B, Top: Youden plot; the solid line depicts the identity line and the broken lines the mass fraction determined by the organizing laboratory; the shaded area is the expanded uncertainty of the mass fraction determination by the organizing laboratory; **Bottom:** Mean & Range plot; the solid line depicts the overall mean, the error bars the range of the blind duplicates; red colour indicates post-column derivatisation, black pre-column, and green LC-MS; non-compliant results are depicted by a "x".

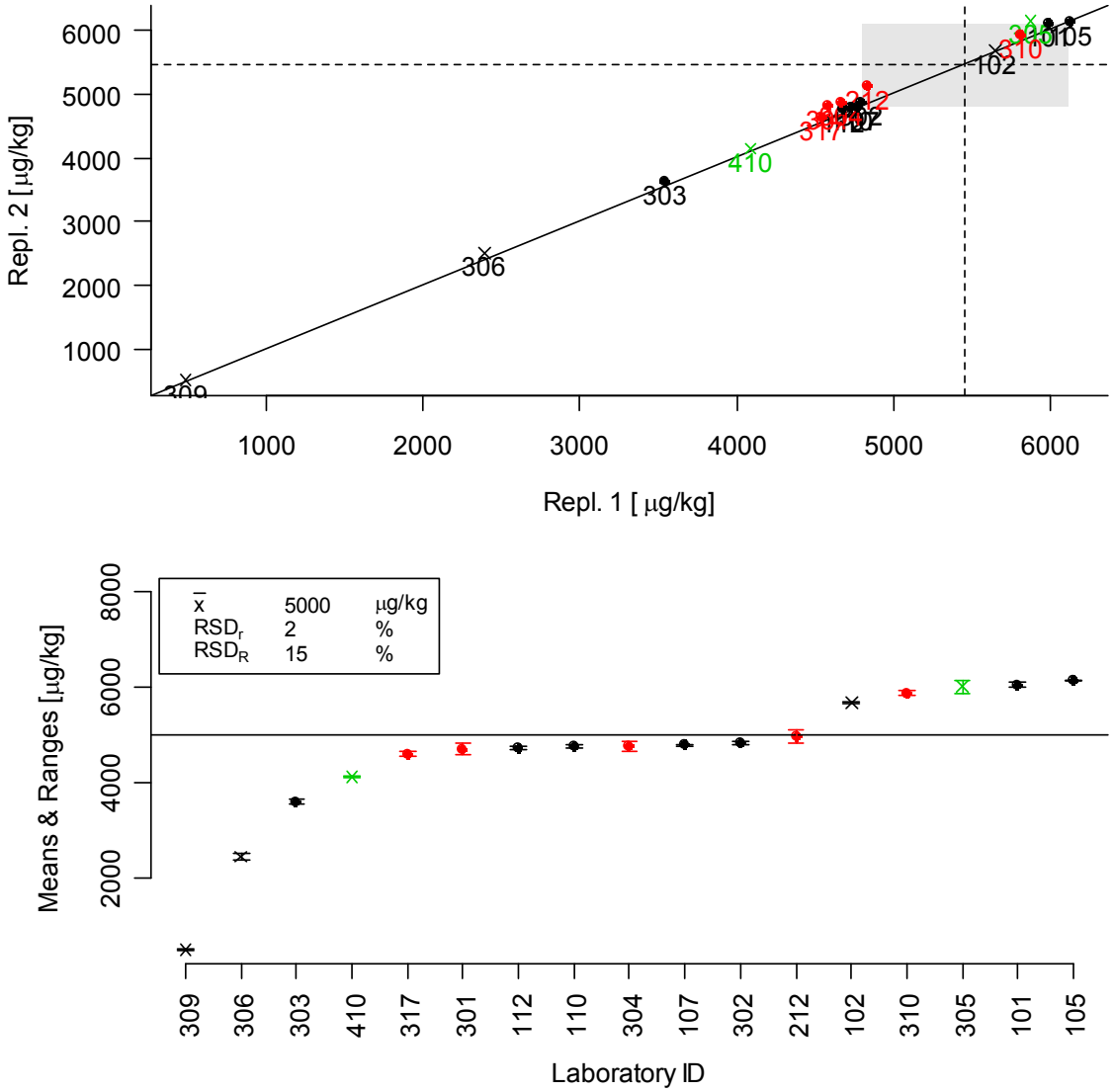

Figure 2, Spike B, Top: Youden plot; the solid line depicts the identity line and the broken lines the mass fraction determined by the organizing laboratory; the shaded area is the expanded uncertainty of the mass fraction determination by the organizing laboratory; **Bottom:** Mean & Range plot; the solid line depicts the overall mean, the error bars the range of the blind duplicates; red colour indicates post-column derivatisation, black pre-column, and green LC-MS; non-compliant results are depicted by a "x".

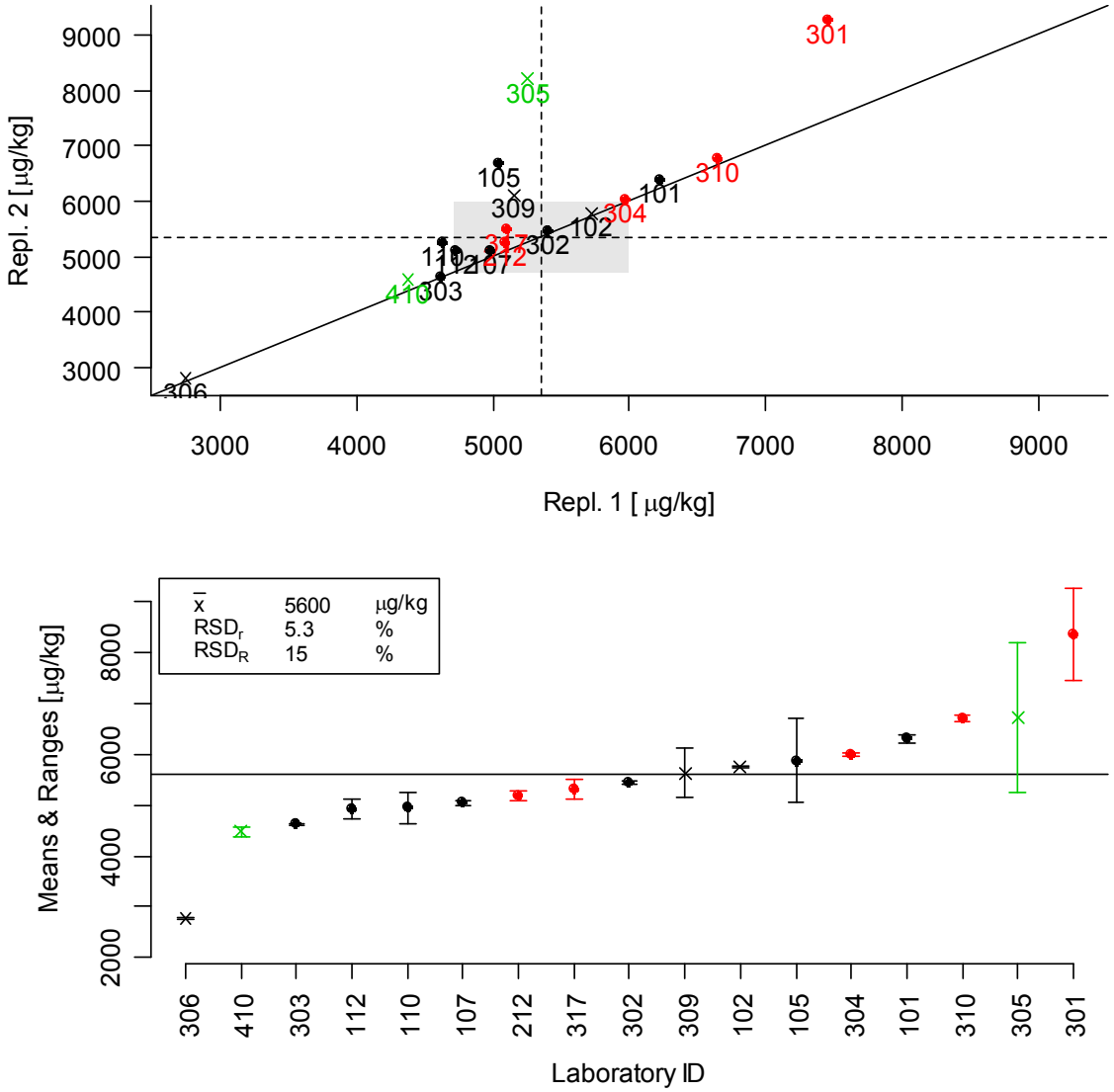

Figure 3, Level C, Top: Youden plot; the solid line depicts the identity line and the broken lines the mass fraction determined by the organizing laboratory; the shaded area is the expanded uncertainty of the mass fraction determination by the organizing laboratory; **Bottom:** Mean & Range plot; the solid line depicts the overall mean, the error bars the range of the blind duplicates; red colour indicates post-column derivatisation, black pre-column, and green LC-MS; non-compliant results are depicted by a "x".

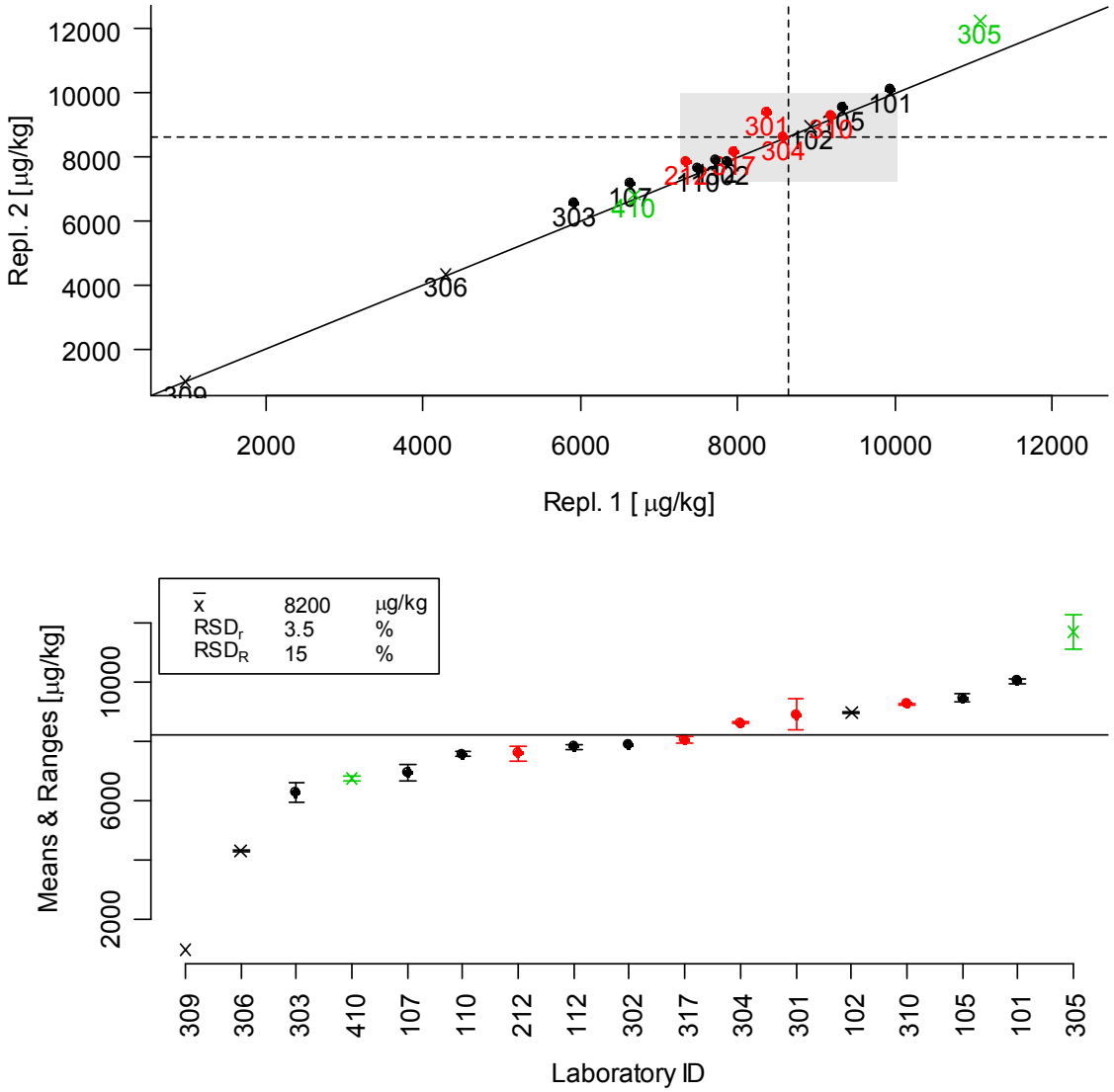

Figure 4, Spike A, Top: Youden plot; the solid line depicts the identity line and the broken lines the mass fraction determined by the organizing laboratory; the shaded area is the expanded uncertainty of the mass fraction determination by the organizing laboratory; **Bottom:** Mean & Range plot; the solid line depicts the overall mean, the error bars the range of the blind duplicates; red colour indicates post-column derivatisation, black pre-column, and green LC-MS; non-compliant results are depicted by a "x".

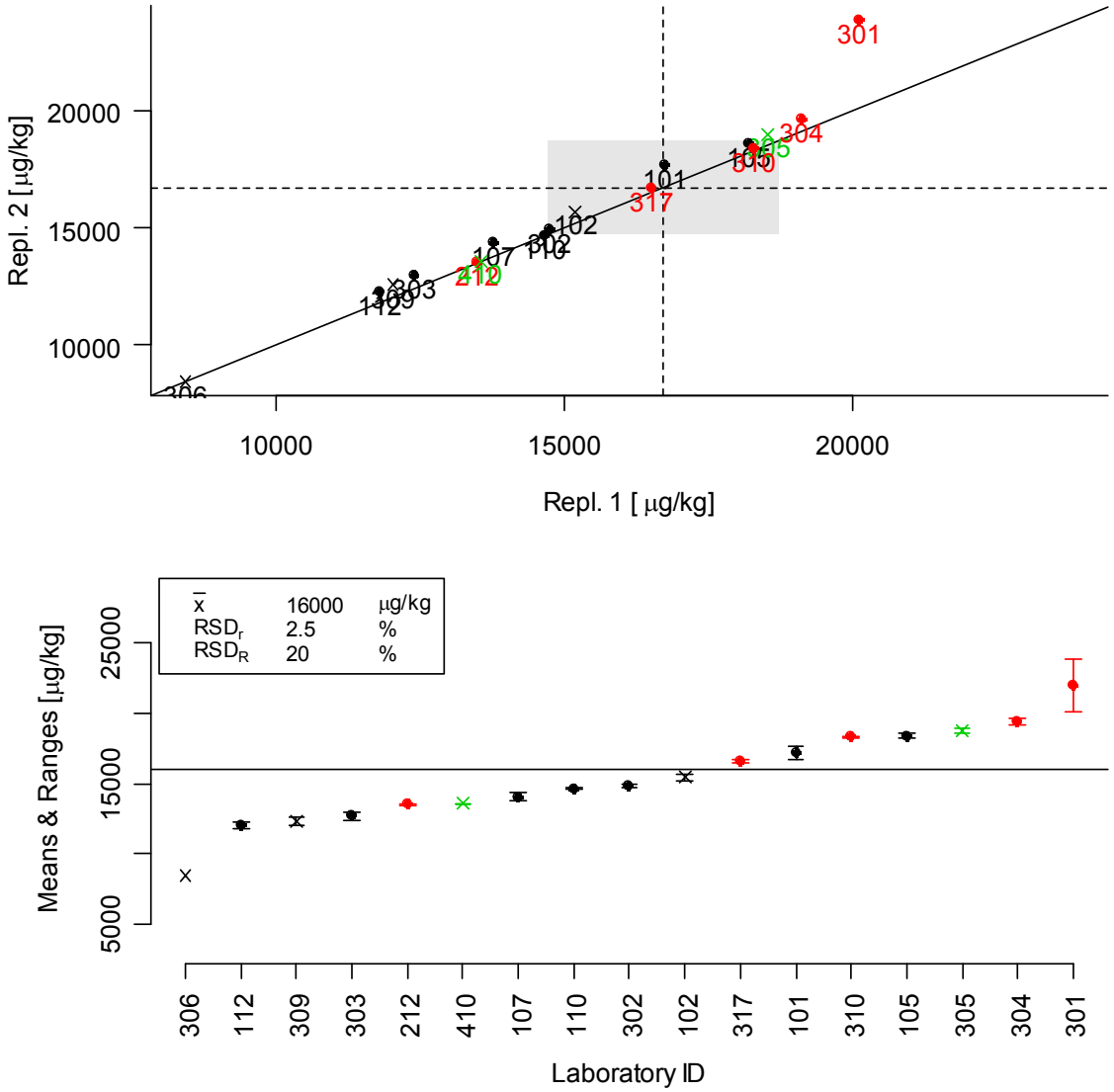

Figure 5, Level SH, Top: Youden plot; the solid line depicts the identity line and the broken lines the mass fraction determined by the organizing laboratory; the shaded area is the expanded uncertainty of the mass fraction determination by the organizing laboratory; **Bottom:** Mean & Range plot; the solid line depicts the overall mean, the error bars the range of the blind duplicates; red colour indicates post-column derivatisation, black pre-column, and green LC-MS; non-compliant results are depicted by a "x".

Since robust methods were used to determine repeatability and reproducibility the results of laboratory 301 were not excluded from the evaluation. However, the inclusion of these results is the main driver for a significantly higher mean of the post-column results compared to the pre-column results. Without the influence of Laboratory 301 pre- and post-column derivatisation would not be different.

Something that stands out from all graphs is that the ratio of repeatability to reproducibility is much smaller than one would normally expect. That causes the data to be so drawn-out in the Youden plots. Since the homogeneity of the materials was demonstrated we believe that the immunoaffinity columns might be the cause. It appears that as long as one preparation of buffers and solvents is used within a limited time period within one laboratory precision is rather good. But as soon as different environmental conditions, buffers, and operators are involved precision degrades quickly. Any laboratory setting up uncertainty budgets for Fumonisin analyses should account for this properly.

A questionnaire was filled-in by all of the 16 laboratories which reported results with questions about their experience, the adequacy of the method description, and, if any, deviations from the method protocol.

Every laboratory claimed previous experience with the determination of Fumonisins, in the majority of the cases it was more than 12 months (13 labs). Two laboratories claimed an experience of 3 to 12 months and only one said they had less than three months of experience. Whether the description of the method was adequate was answered with "Yes" by 13 laboratories.

Of the three laboratories which did not answer with "Yes" one remarked (i) that the "batch of runs as described is too long" and that the need for a sufficient supply of pre-column derivatisation reagent was not mentioned. The second laboratory remarked (ii) that the actual concentrations of the calibration solutions should be stated and the calibration range should be aimed at maximum recommended levels for feed. Furthermore, it remarked that the reaction time for the post-column derivatisation set-up as described was very short. The third laboratory remarked (iii) that the "freshness" of the pre-column derivatisation reagent was of importance and that the permissible age of the reagent was not stated.

Our reply is: (i) it is important from our experience to bracket samples between calibration samples and to run samples and calibration solutions at least in duplicate in changing order. The reason is to account for drifts of the system and possible interactions between samples of widely differing concentrations. For the purpose of this study this resulted in a long sequence. It was up to the individual participant to make provisions for a sufficient supply of reagent since we are unable to make recommendations suitable for all the different instrumentations possibly used.

(ii) We did not state concentrations because FB_1 & FB_2 stock solutions of different concentrations were being used and that would have made for a very complex table. The calibration range was aimed at maximum recommended levels in feed since it covered the mass fraction range of 0.2 to 40 µg/kg for the individual Fumonisin and, hence, 0.4 to 80 µg/kg for the sum of FB_1 & FB_2. And with regards to the shortness of the reaction time in the described post-column derivatisation set-up: it had to be a compromise of sufficient reaction time and minimal band broadening due to extra-column volumes and it worked well in our laboratory.

(iii) We agree and failed to mention in Sec. 4.19.2 of the method protocol that the pre-column reagent had to be prepared freshly for every batch of samples as it was mentioned for the post-column reagent.

A very important question was whether at any step of the protocol sections 4 ("Reagents and Materials"), 5 ("Spiking procedure"), and 6 ("Sample preparation") there were any deviations. 11 laboratories answered with "No" and five with "Yes". Of the five laboratories which answered "Yes" (i) one did not perform the verification of a result above 10000 µg/kg by preparing and measuring an additional dilution of the raw extract (method protocol Sec. 6.1); (ii) one used disodium hydrogen phosphate dihydrate instead of disodium hydrogen phosphate dodecahydrate with properly adjusted weight (method protocol Sec. 4.6); (iii) one used volumetric flasks of volumes different from the ones described (method protocol Sec. 4.22) with properly adjusting the volumes of diluted stock solution; (iv) one used "back flushing" for the elution (method protocol Sec. 6.2) as described in the instructions of the immunoaffinity columns while maintaining all volumes;

(v) one filtered the extract before dilution (method protocol Sec. 6.1) instead of waiting for it to settle.

Deviations (ii), (iii), and (iv) were considered to be non-essential and therefore permissible. Deviation (v) was performed by Laboratory 102 which also deviated from the prescribed mobile phase additive which led to its exclusion. Deviation (i) was a disregard of the recommendation to verify results with mass fractions above 10000 µg/kg which was made to safeguard against overloading of the immunoaffinity columns. It did not lead to exclusion of the respective laboratory because the results of the affected material did not show an unusual bias as it would be expected if the capacity of the immunoaffinity column had been exceeded.

Fourteen of the laboratories reported a time range of 20 to 40 minutes for the immunoaffinity clean-up step, one laboratory reported less than 20 min, and one more than 40 min.

Whether deviations from the method protocol section 7 "Measurements" were made was also a question. Surprisingly three laboratories answered this question with "No". Since these three laboratories used equipment different from the one for which the recommendations were made it must be seen as incidental that the separation requirements as set out in the protocol (Sec 3.13.3) were met. One of the remaining 13 laboratories changed the mobile phase additive which was a clear non-compliance. The other 12 laboratories introduced changes to the isocratic or gradient conditions and/or the autosampler settings in order to achieve acceptable separation as was recommended in the protocol.

8. CONCLUSIONS

It could be shown that the method under investigation performed satisfactorily. Evaluation of the reports of 16 laboratories from 12 Member States of the European Union resulted, after exclusion of 4 non-compliant laboratories, in relative reproducibility standard deviations of 15 % to 20% for contamination levels from 3 mg/kg to 16 mg/kg for the sum of Fumonisin B_1 and B_2. The Horwitz ratios of 1.2 to 1.9 were all within the acceptable range and even performance criteria set out for food analysis [6] were met by this method of analysis for animal feed.

The compliant measurements were performed using pre-column derivatisation by seven laboratories and post-column derivatisation by five laboratories. The performances of pre- and post-column derivatisation must be seen as comparable. The description of the method was judged as adequate by the majority of the laboratories.

We consider this method as fit for the purpose of enforcing maximum recommended levels of the sum of Fumonisin B_1 and B_2 in animal compound feed and maize intended for animal feed [9] and have submitted it to the European Committee for Standardisation (CEN)/ Technical Committee (TC) 327 Animal feeding stuffs.

9. ACKNOWLEDGEMENTS

We want to thank Carsten Mischke for his most valuable technical support in preparing the test materials. Thanks also go out to Anne-Mette Jensen and Franz Ulberth for reviewing and improving the document.

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11. ANNEX A

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Dr. Benedikt	Brand	Staatliches Veterinäruntersuchungsamt Arnsberg Dezernat 43 - Verbraucherschutzprogramm, Futtermittel	Zur Taubeneiche 10-12	D-59821	Arnsberg	Germany
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First Name	Surname	Company/Institute	Address	Postal Code	Town	Country (full)
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Sona	Tkacikova	State Veterinary and Food Institute Košice	Hlinkova 1	043 65	Košice	Slovak Republic
Jeroen	Vancutsem	Federaal Agentschap voor de Veiligheid van de Voedselketen (FAVV)	Leuvensesteenweg 17	B-3080	Tervuren	Belgium
Yveta	Vojsova	State Veterinary and Food Institute Bratislava Department of Chemistry and Toxicology	Botanicka 15	842 52	Bratislava	Slovak Republic

12. ANNEX B

The result tables and graphs for the individual fumonisins:

Table B. 1: Mass fractions of FB_1 in µg/kg for the blind duplicates of the five materials by laboratory. Spike A consists of Level C plus 2410 µg/kg FB_1, Spike B of Level B plus 2009 µg/kg FB_1. Grey shading indicates exclusion because of non-compliance.

Lab ID	Spike A		Spike B		Level B		Level C		Level SH	
101	8029	7950	4788	4710	2821	2818	5033	5181	14390	13584
102	7256	7218	4573	4550	2533	2590	4757	4807	12468	12843
105	7902	7693	4926	4908	2793	2841	4141	5507	15879	15546
107	6018	5528	3977	3977	2345	2238	4384	4274	11536	12017
110	6437	6281	3933	3979	2409	2339	4512	3945	12513	12522
112	6710	6548	4029	3967	2372	2403	4454	4346	10183	10564
212	6032	6497	4181	3965	2478	2462	4460	4284	11286	11237
301	6383	7251	3398	3370	3195	2826	5605	4647	16124	14877
302	6441	6421	3991	3944	2455	2441	4647	4595	12284	12096
303	5638	5034	3056	3121	1856	1985	4019	3942	11036	10584
304	6805	6841	3631	3773	2625	2724	4907	4794	14631	15055
305	9559	8812	4862	4665	3191	2734	6712	4343	15098	15626
306	3764	3750	2208	2092	1177	1142	2509	2513	7219	7265
309	542	528	163	165	2013	2176	4090	5054	9730	10246
310	7620	7529	4753	4823	3131	3197	5588	5705	15301	15245
317	6720	6396	3738	3894	2398	2402	4450	4294	13777	13564
410	5618	5507	3397	3367	2106	2068	3888	3703	11393	11345

Table B. 2: Method performance parameters for FB_1 in ascending order of the mean; Spike B consists of Level B plus 2009 µg/kg FB_1, Spike A of Level C plus 2410 µg/kg FB_1; the apparent recoveries were calculated based on these additions; n.a. – not applicable.

Performance parameter	Level B	Spike B	Level C	Spike A	Level SH
Mean value $\overline{x}$ [µg/kg]	2490	3920	4590	6520	12700
Repeatability standard deviation s_r [µg/kg]	68.8	69.4	274	239	307
Relative repeatability standard deviation RSD_r [%]	2.8	1.8	6	3.7	2.4

Performance parameter	Level B	Spike B	Level C	Spike A	Level SH
Repeatability limit *r* [µg/kg]	193	194	766	669	861
Reproducibility standard deviation s_R [µg/kg]	432	789	630	1240	2410
Relative reproducibility standard deviation RSD_R [%]	17	20	14	19	19
Horwitz ratio	1.2	1.5	1.1	1.6	1.7
Reproducibility limit *R* [µg/kg]	1210	2210	1760	3470	6750
Apparent recovery [%]	n.a.	71	n.a.	80	n.a.

Table B. 3: Mass fractions of FB_2 in µg/kg for the blind duplicates of the five materials by laboratory. Spike A consists of Level C plus 877 µg/kg FB_2, Spike B of Level B plus 731 µg/kg FB_2. Grey shading indicates exclusion because of non-compliance.

Lab ID	Spike A		Spike B		Level B		Level C		Level SH	
101	2074	2001	1311	1284	725	725	1189	1213	3290	3151
102	1720	1711	1104	1097	505	513	964	973	2723	2838
105	1669	1647	1214	1224	633	685	903	1191	2697	2633
107	1189	1107	795	808	361	339	715	709	2249	2336
110	1209	1201	801	802	465	463	745	686	2147	2124
112	1178	1171	738	713	344	362	664	380	1608	1736
212	1318	1351	932	872	464	459	806	809	2254	2243
301	1992	2168	1415	1211	1427	1412	1857	4610	7684	5227
302	1433	1448	862	851	413	387	835	814	2676	2632
303	957	885	491	517	269	390	617	675	1924	1832
304	1778	1789	1037	1086	756	773	1123	1180	4475	4603
305	2708	2265	1287	1210	695	616	1498	913	3426	3347
306	588	535	295	298	0	0	293	234	1223	1175
309	437	452	320	343	490	499	1063	1059	2303	2331
310	1670	1668	1058	1106	576	599	1062	1074	3086	3032
317	1442	1556	800	744	443	498	1044	812	2917	2934
410	1210	1176	744	722	374	379	696	675	2185	2205

Table B. 4: Method performance parameters for FB_2 in ascending order of the mean; Spike B consists of Level B plus 731 µg/kg FB_2, Spike A of Level C plus 877 µg/kg FB_2; the apparent recoveries were calculated based on these additions; n.a. – not applicable.

Performance parameter	Level B	Spike B	Level C	Spike A	Level SH
Mean value $\bar{x}$ [µg/kg]	507	893	903	1430	2610
Repeatability standard deviation s_r [µg/kg]	20.3	29.3	102	44.3	66.5
Relative repeatability standard deviation RSD_r [%]	4	3.3	11	3.1	2.5
Repeatability limit r [µg/kg]	56.9	82	286	124	186
Reproducibility standard deviation s_R [µg/kg]	188	340	298	535	779
Relative reproducibility standard deviation RSD_R [%]	37	38	33	37	30
Horwitz ratio	2.1	2.3	2.0	2.5	2.1
Reproducibility limit R [µg/kg]	526	952	835	1500	2180
Apparent recovery [%]	n.a.	53	n.a.	60	n.a.

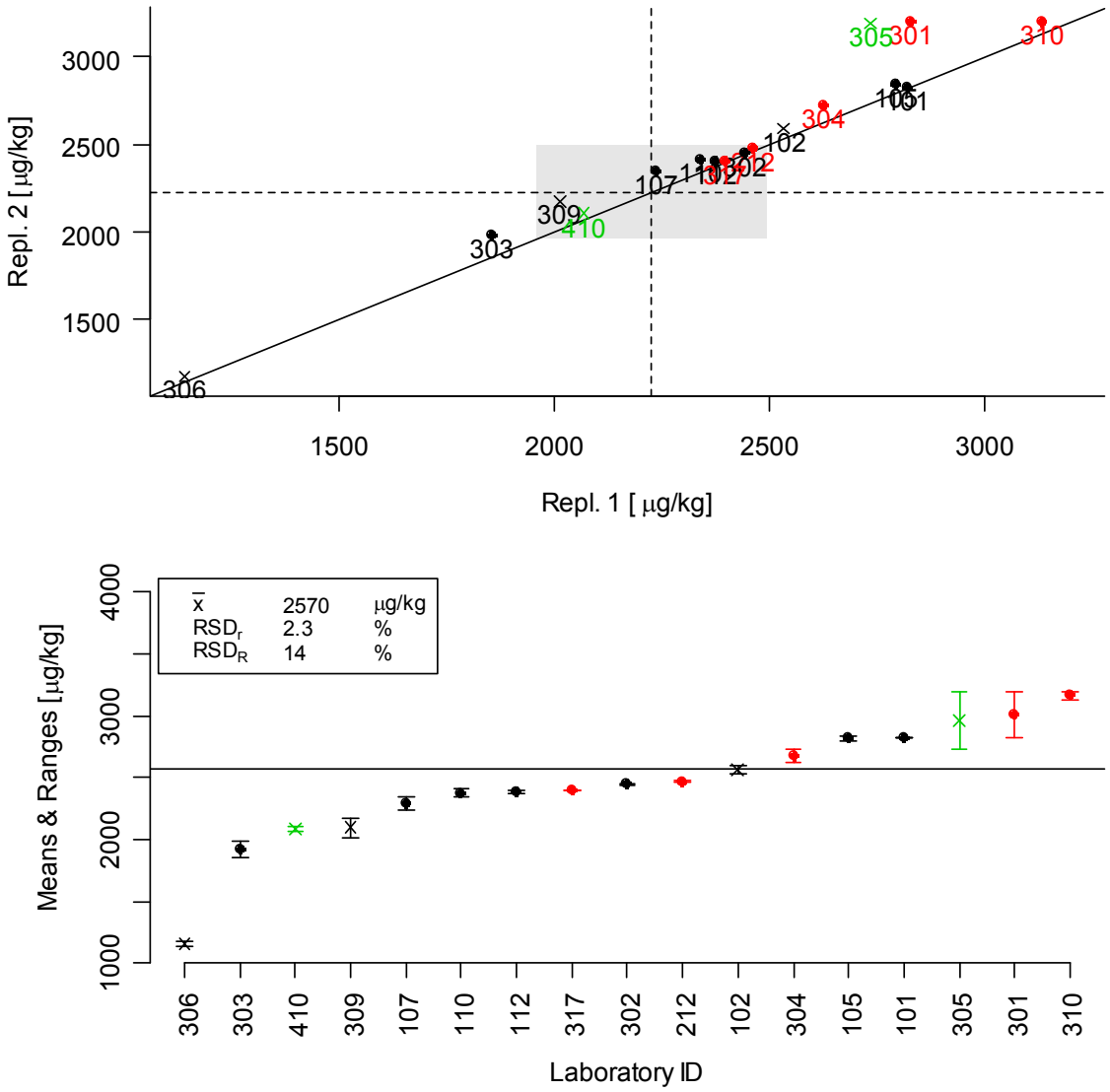


Figure B. 1, Level B FB_1, Top: Youden plot; the solid line depicts the identity line and the broken lines the mass fraction determined by the organizing laboratory; the shaded area is the expanded uncertainty of the mass fraction determination by the organizing laboratory; **Bottom:** Mean & Range plot; the solid line depicts the overall mean, the error bars the range of the blind duplicates; red colour indicates post-column derivatisation, black pre-column, and green LC-MS; non-compliant results are depicted by a "x".

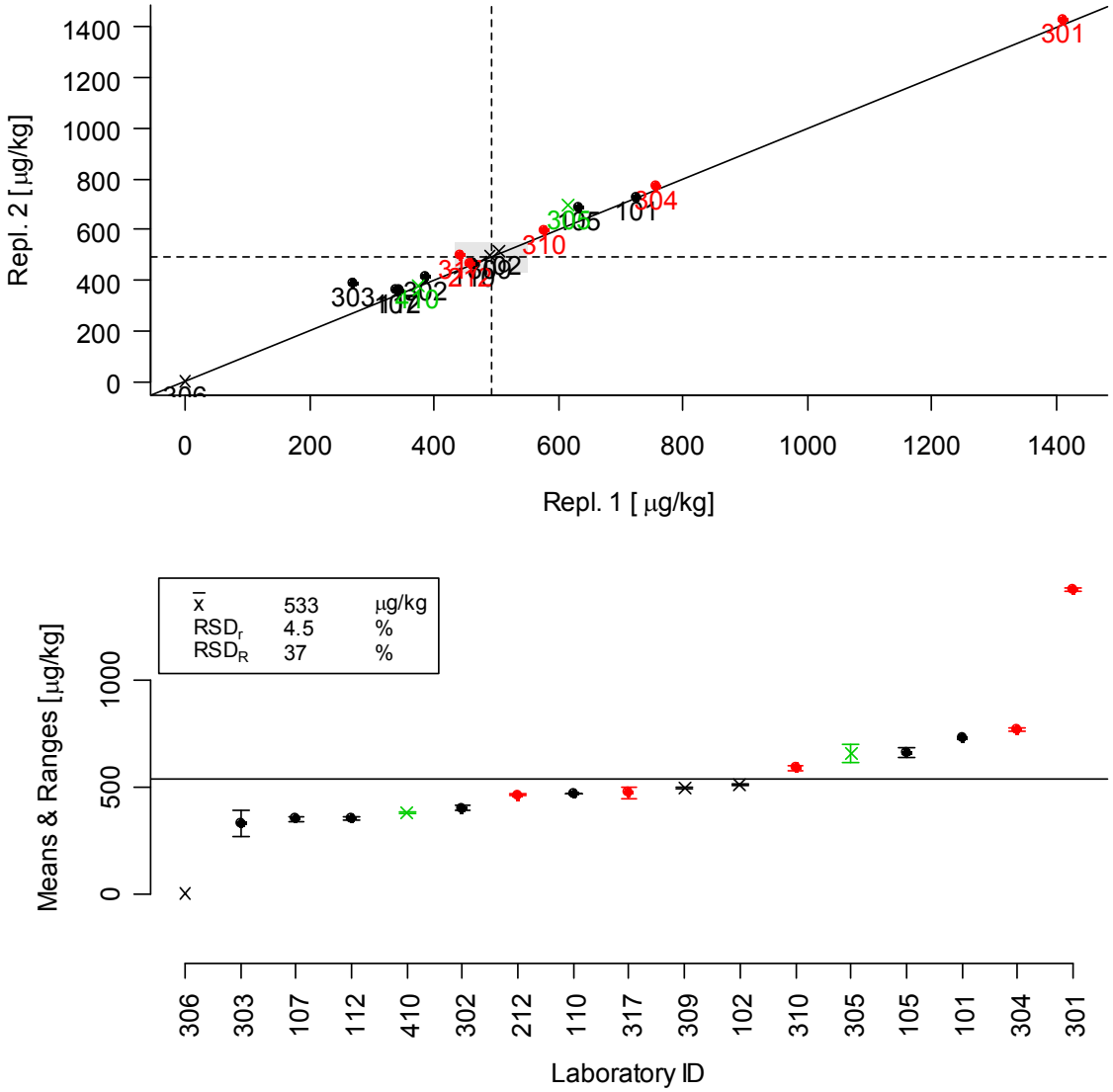


Figure B. 2, Level B FB$_2$, Top: Youden plot; the solid line depicts the identity line and the broken lines the mass fraction determined by the organizing laboratory; the shaded area is the expanded uncertainty of the mass fraction determination by the organizing laboratory; **Bottom:** Mean & Range plot; the solid line depicts the overall mean, the error bars the range of the blind duplicates; red colour indicates post-column derivatisation, black pre-column, and green LC-MS; non-compliant results are depicted by a "x".

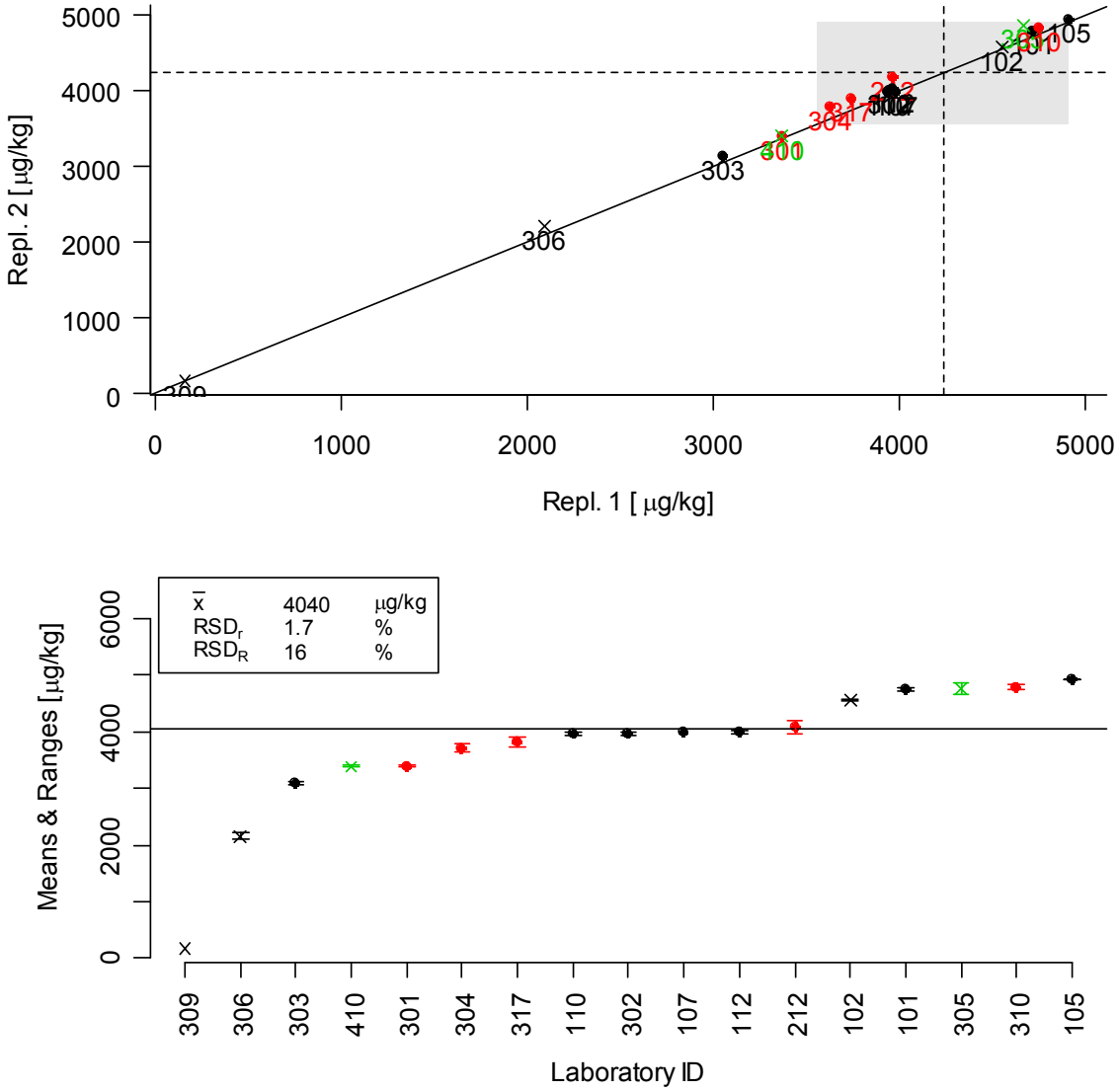


Figure B. 3, Spike B FB$_1$, Top: Youden plot; the solid line depicts the identity line and the broken lines the mass fraction determined by the organizing laboratory; the shaded area is the expanded uncertainty of the mass fraction determination by the organizing laboratory; **Bottom:** Mean & Range plot; the solid line depicts the overall mean, the error bars the range of the blind duplicates; red colour indicates post-column derivatisation, black pre-column, and green LC-MS; non-compliant results are depicted by a "x".

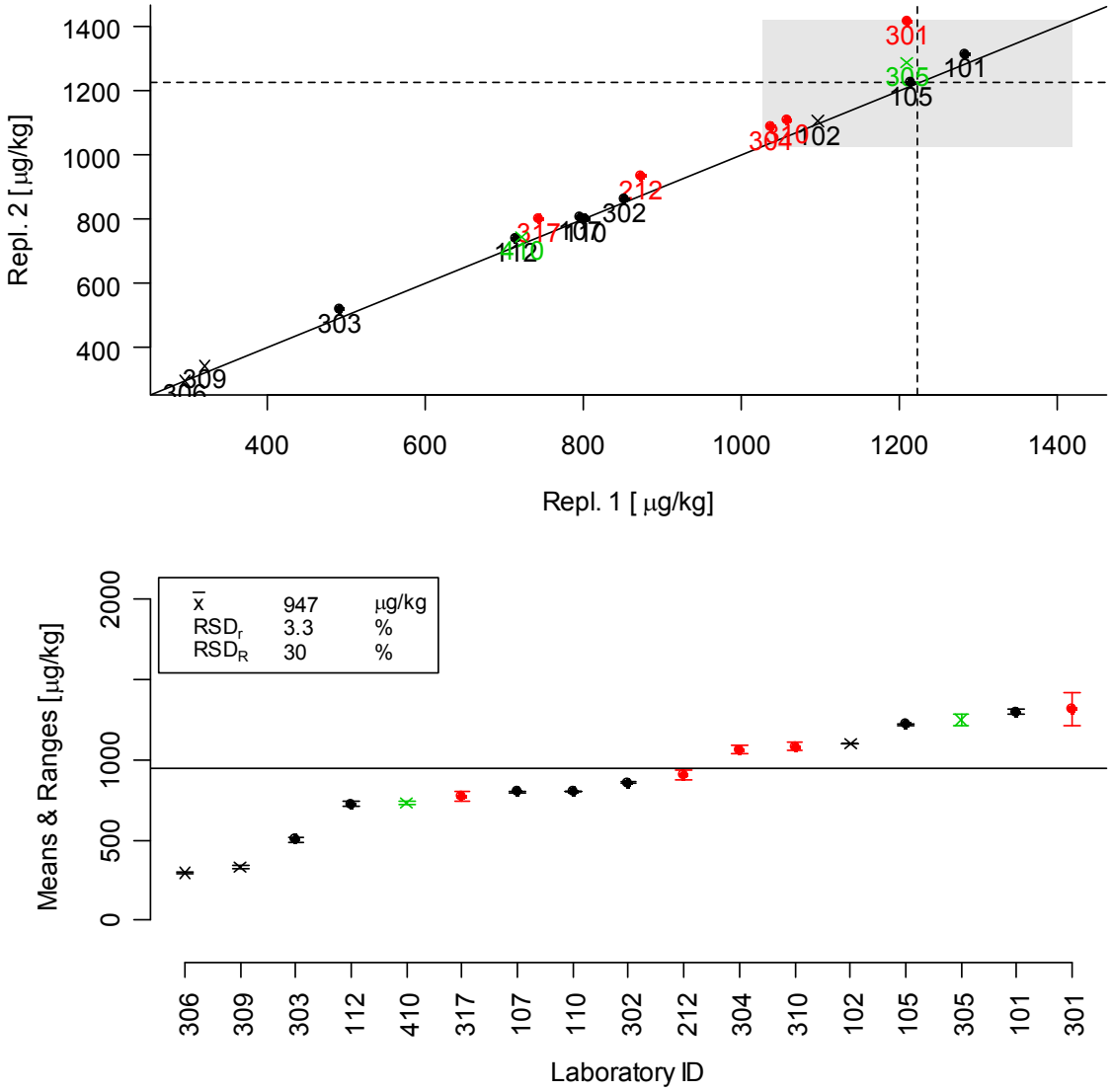


Figure B. 4, Spike B FB_2, Top: Youden plot; the solid line depicts the identity line and the broken lines the mass fraction determined by the organizing laboratory; the shaded area is the expanded uncertainty of the mass fraction determination by the organizing laboratory; **Bottom:** Mean & Range plot; the solid line depicts the overall mean, the error bars the range of the blind duplicates; red colour indicates post-column derivatisation, black pre-column, and green LC-MS; non-compliant results are depicted by a "x".

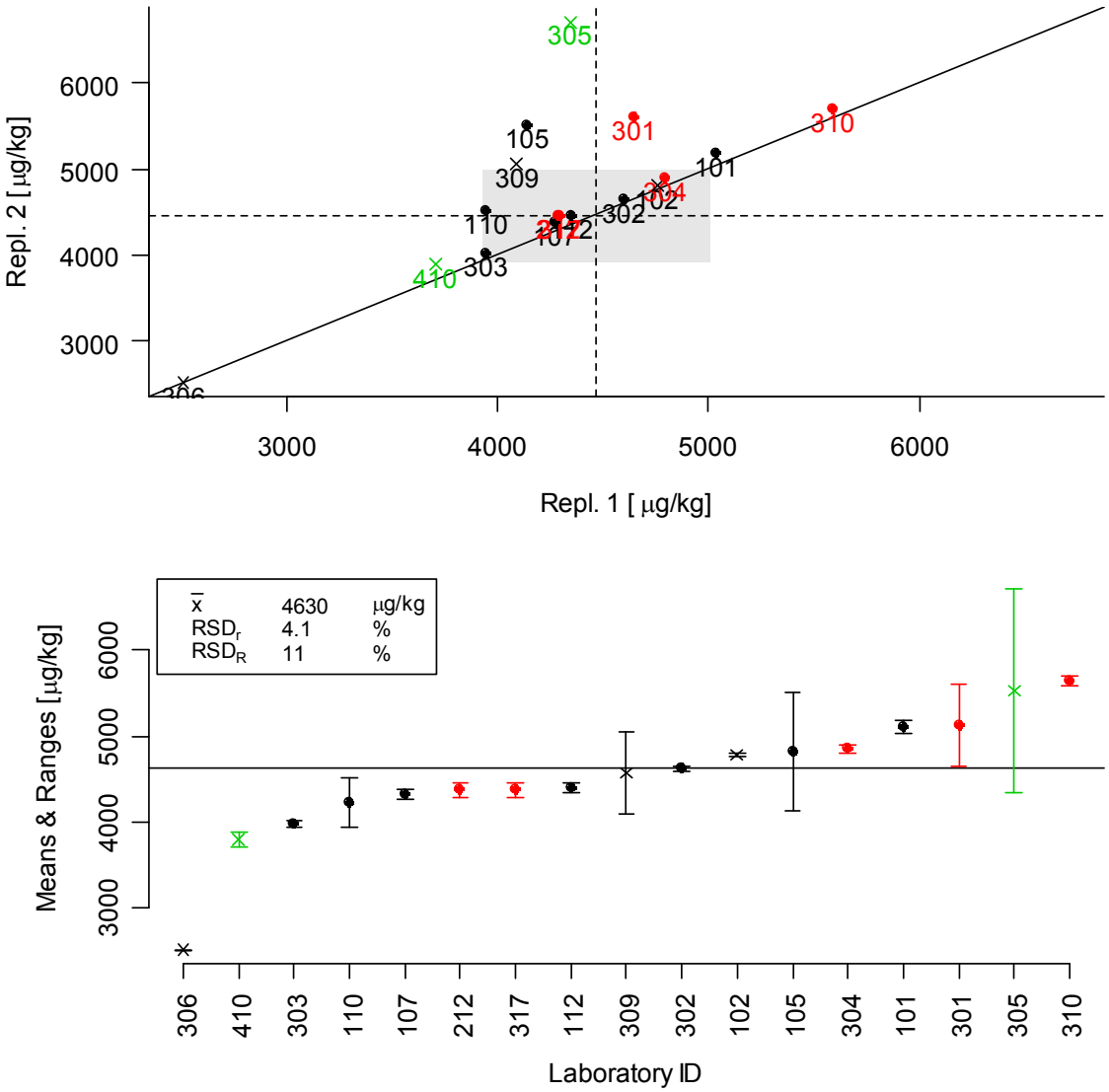


Figure B. 5, Level C FB_1, Top: Youden plot; the solid line depicts the identity line and the broken lines the mass fraction determined by the organizing laboratory; the shaded area is the expanded uncertainty of the mass fraction determination by the organizing laboratory; **Bottom:** Mean & Range plot; the solid line depicts the overall mean, the error bars the range of the blind duplicates; red colour indicates post-column derivatisation, black pre-column, and green LC-MS; non-compliant results are depicted by a "x".

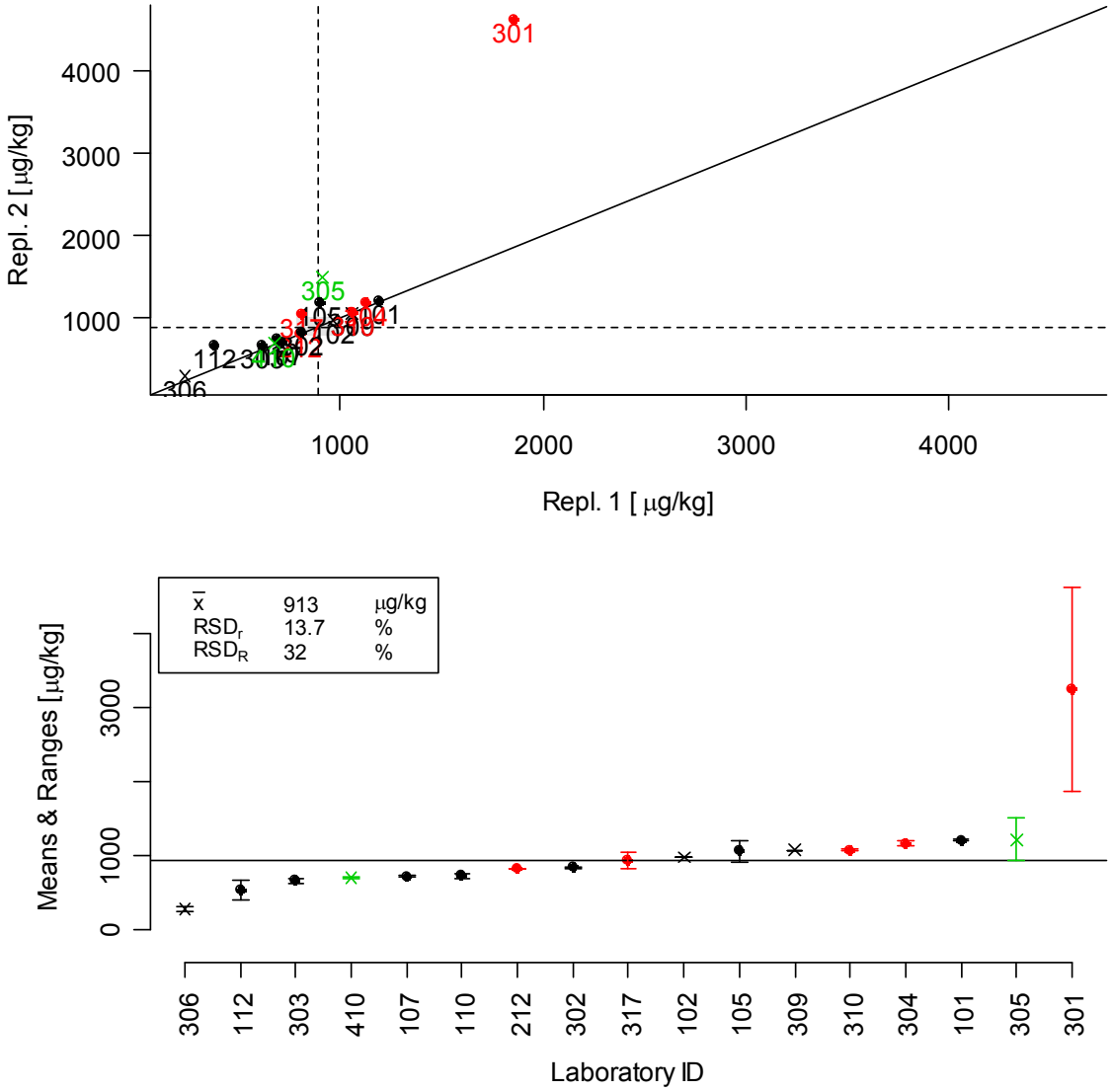


Figure B. 6, Level C FB_2, Top: Youden plot; the solid line depicts the identity line and the broken lines the mass fraction determined by the organizing laboratory; the shaded area is the expanded uncertainty of the mass fraction determination by the organizing laboratory; **Bottom:** Mean & Range plot; the solid line depicts the overall mean, the error bars the range of the blind duplicates; red colour indicates post-column derivatisation, black pre-column, and green LC-MS; non-compliant results are depicted by a "x".

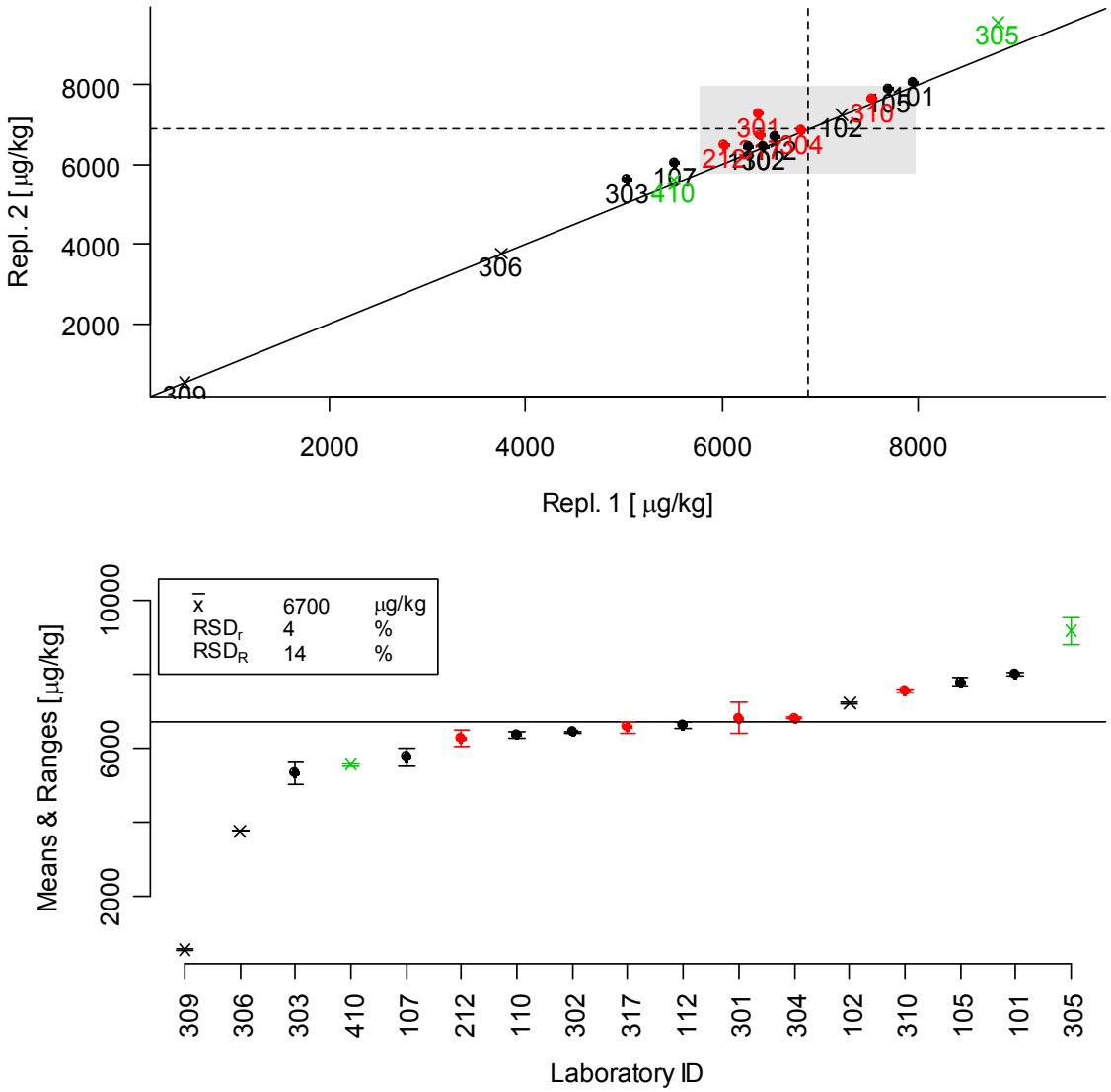


Figure B. 7, Spike A FB_1, Top: Youden plot; the solid line depicts the identity line and the broken lines the mass fraction determined by the organizing laboratory; the shaded area is the expanded uncertainty of the mass fraction determination by the organizing laboratory; **Bottom:** Mean & Range plot; the solid line depicts the overall mean, the error bars the range of the blind duplicates; red colour indicates post-column derivatisation, black pre-column, and green LC-MS; non-compliant results are depicted by a "x".

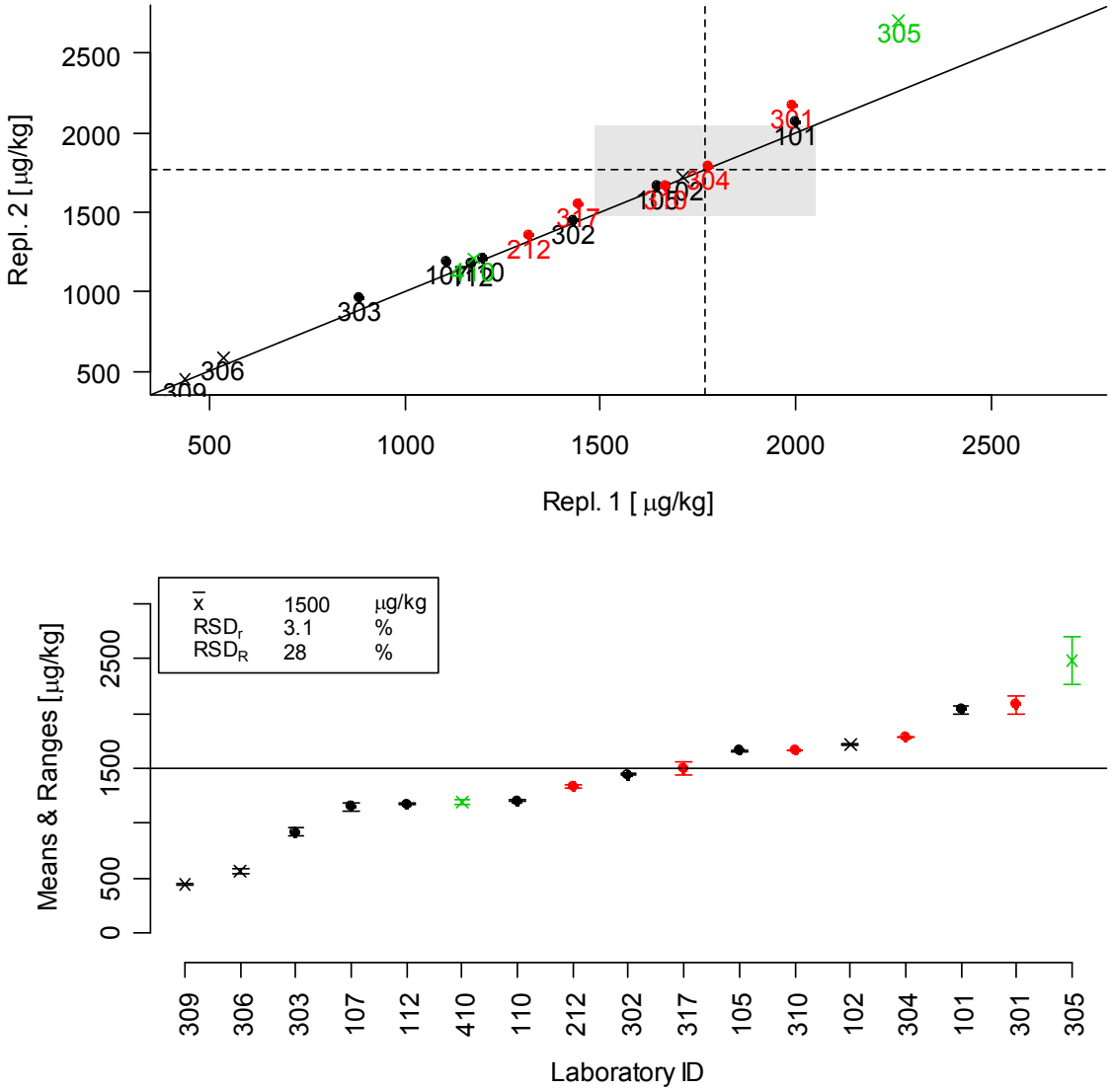


Figure B. 8, Spike A FB_2, Top: Youden plot; the solid line depicts the identity line and the broken lines the mass fraction determined by the organizing laboratory; the shaded area is the expanded uncertainty of the mass fraction determination by the organizing laboratory; **Bottom:** Mean & Range plot; the solid line depicts the overall mean, the error bars the range of the blind duplicates; red colour indicates post-column derivatisation, black pre-column, and green LC-MS; non-compliant results are depicted by a "x".

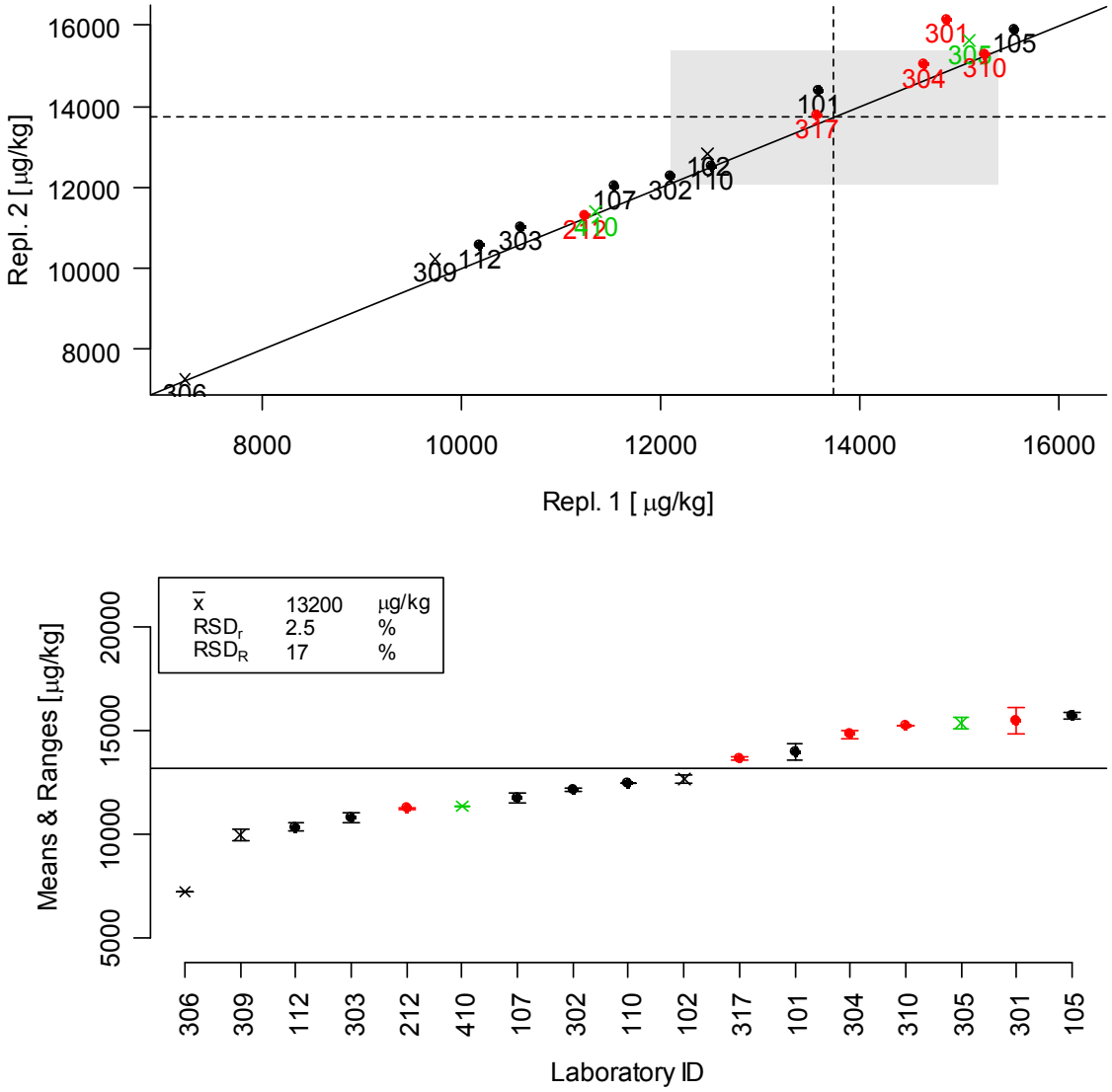


Figure B. 9, Level SH FB$_1$, Top: Youden plot; the solid line depicts the identity line and the broken lines the mass fraction determined by the organizing laboratory; the shaded area is the expanded uncertainty of the mass fraction determination by the organizing laboratory; **Bottom:** Mean & Range plot; the solid line depicts the overall mean, the error bars the range of the blind duplicates; red colour indicates post-column derivatisation, black pre-column, and green LC-MS; non-compliant results are depicted by a "x".

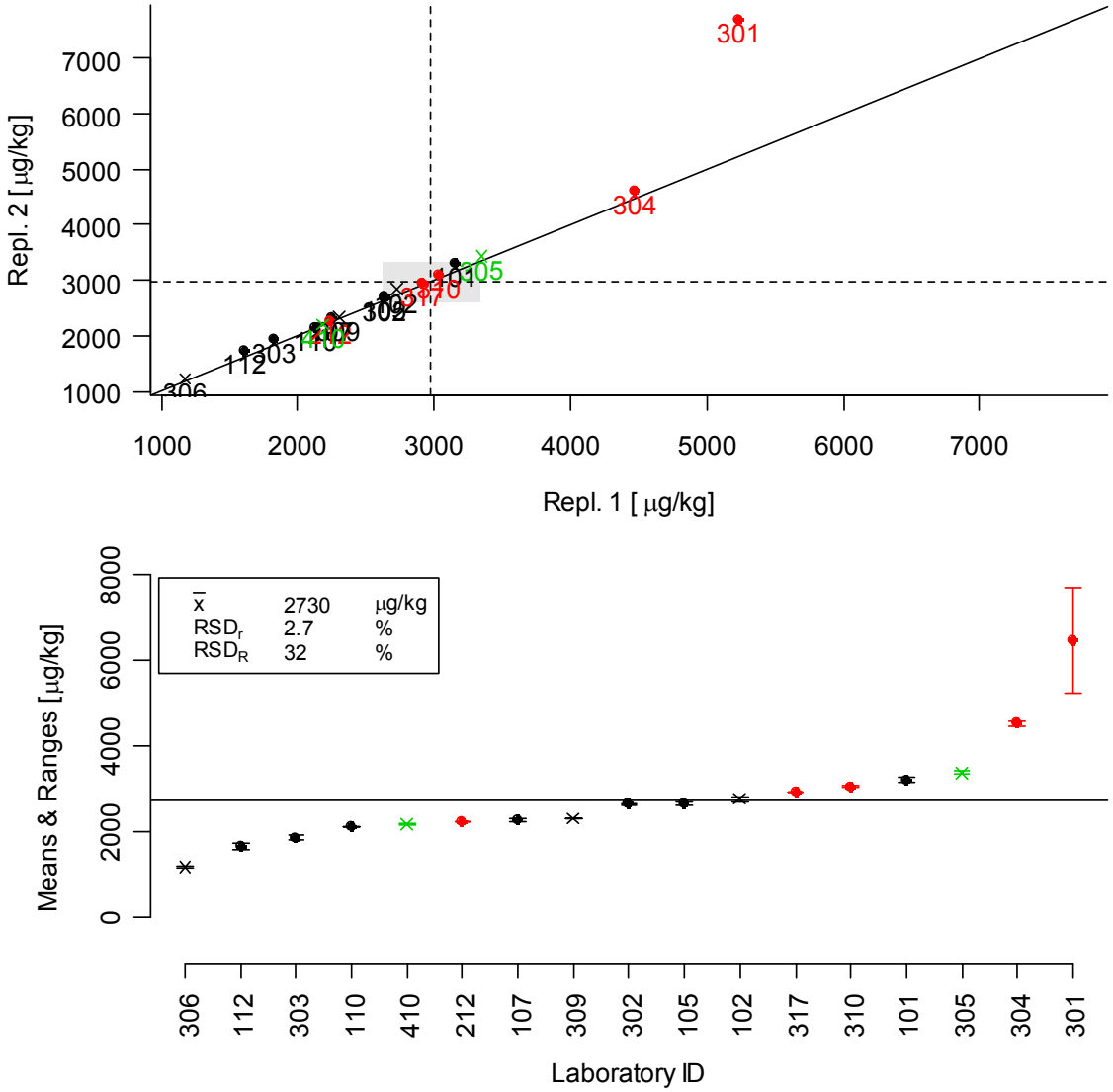


Figure B. 10, Level SH FB_2, Top: Youden plot; the solid line depicts the identity line and the broken lines the mass fraction determined by the organizing laboratory; the shaded area is the expanded uncertainty of the mass fraction determination by the organizing laboratory; **Bottom:** Mean & Range plot; the solid line depicts the overall mean, the error bars the range of the blind duplicates; red colour indicates post-column derivatisation, black pre-column, and green LC-MS; non-compliant results are depicted by a "x".

13. ANNEX C

Method protocol:

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements
Food safety and quality

Determination of the Sum of Fumonisin B_1 & B_2 in compound animal feed with immunoaffinity clean-up and RP-HPLC with fluorescence detection after pre-column derivatisation (optional post-column derivatisation)

Contents

Foreword

READ THIS PROTOCOL CLOSELY! THERE HAVE BEEN CHANGES TO PREVIOUS VERSIONS YOU MIGHT HAVE RECEIVED.

THIS IS A STUDY OF THE METHOD, NOT OF THE LABORATORY. THE METHOD MUST BE FOLLOWED AS CLOSELY AS PRACTICABLE, AND ANY DEVIATIONS FROM THE METHOD AS DESCRIBED, NO MATTER HOW TRIVIAL THEY MAY SEEM, MUST BE NOTED ON THE REPORT FORM AND MAY LEAD TO EXCLUSION FROM THE EVALUATION PHASE.

SOME OF THE HPLC CONDITIONS ARE JUST RECOMMENDATIONS WHEREAS OTHERS ARE BINDING. READ SECTION 7.1 CAREFULLY! DO NOT START WITH THE PREPARATION OF THE MATERIALS UNTIL YOU HAVE SUFFICIENT RETENTION AND RESOLUTION.

SECTION 4.23 "Immunoaffinity columns" HAS NO BEARING FOR THIS TRIAL AND IS MEANT FOR FUTURE REFERENCE.

BE AWARE THAT SOME MATERIALS COULD BE HIGHLY CONTAMINATED (SEE PROVISIONS IN SEC. 6.1 AND 8).

***WARNING* — THE USE OF THIS PROTOCOL INVOLVES HAZARDOUS MATERIALS, OPERATIONS AND EQUIPMENT. THIS PROTOCOL DOES NOT PURPORT TO ADDRESS ALL THE SAFETY PROBLEMS ASSOCIATED WITH ITS USE. IT IS THE RESPONSIBILITY OF THE USER OF THIS PROTOCOL TO ESTABLISH APPROPRIATE SAFETY AND HEALTH PRACTICES AND DETERMINE THE APPLICABILITY OF REGULATORY LIMITATIONS PRIOR TO USE.**

1. SCOPE

This protocol specifies a candidate method for further standardisation for the determination of Fumonisin B_1 & B_2 (FB_1 & FB_2) in compound animal feed using liquid-chromatography with fluorescence detection after either pre- or post-column derivatisation. This candidate method will be validated for the determination of FB_1 and FB_2 through the analysis of naturally contaminated and spiked compound animal feed.

2. PRINCIPLE

FB_1 and FB_2 are extracted from the test material with a solution of 50% methanol in phosphate-buffered saline. Then the extract is cleaned up using immunoaffinity columns (IAC). FB_1 and FB_2 are eluted from the IAC using first methanol and then water. After volume adjustment the eluate is directly injected into the HPLC.

3. APPARATUS

Normal laboratory equipment and, in particular, the following:

3.1. 250 mL flasks with screw caps

3.2. Flask shaker

3.3. Graduated cylinders

5, 50, 1000, 2000 mL

3.4. Graduated pipettes

2 mL (Class A ± 0.01mL)
10 mL (Class A ± 0.05mL)
50 mL (Class A ± 0.035mL)

3.5. Analytical balance (d= 0.01g)

3.6. Glass micro fibre filter (e.g. Whatman 934-AH4 125 mm)

3.7. Filter funnel, e.g. 11 cm O.D.

3.8. Auto sampler vials with caps

3.9. Reservoirs for immunoaffinity columns

20-50 mL capacity with adapter for connection to top of immunoaffinity columns.

3.10. Volumetric flasks

20 mL (Class A, ± 0.04 mL)
10 mL (Class A, ± 0.04 mL)
5 mL (Class A, ± 0.04 mL)
2 mL (Class A, ± 0.025 mL)

3.11. Gastight glass syringes and/or positive displacement pipettes

Capable of precisely dispensing the following volumes: 5, 50, 125, 160, 500, and 1000µL

3.12. Support stand for immunoaffinity columns (12 mm O.D.)

3.13. HPLC instrumentation, comprising the following:

3.13.1. Solvent delivery system:

13.1.1. capable of generating a binary gradient with sufficient precision at the required pressures, e.g. Agilent Series 1200 pump.

3.13.2. Auto sampler:

13.1.2. capable of injecting sufficient volumes of injection solution with sufficient repeatability and, for pre-column derivatisation, capable of mixing reagent and sample solution before injection, e.g. Agilent Series 1200 ALS.

3.13.3. Chromatographic column:

13.1.3. any column which provides symmetric peak (peak asymmetry factor $0.9 < A_s < 1.4$ at 10% of full height), sufficient retention ($k > 2$), and resolution ($R_s > 1$) for FB_1 & FB_2, e.g. Agilent Zorbax SB-C18 4.6 x 150mm 3.5 µm.

3.13.4. Fluorescence detector:

13.1.4. capable of providing the required excitation and emission wavelengths and equipped with a flow cell of appropriate size, e.g. Agilent Series 1200 FLD/ Waters 474.

3.13.5. Post-column derivatisation system (not necessary if pre-column derivatisation is used):

13.1.5. either a commercial unit (e.g., Pickering Laboratories) or self-assembled. If self-assembled the following is needed:

3.13.5.1. Reagent pump: capable of delivering a constant pulsation-free flow of the derivatisation reagent against the required pressures.

3.13.5.2. Peek tubing: of the outer diameter required by the HPLC system in use and varying inner diameters, e.g. 1/16"OD, 0.04", 0.02" ID, 0.01" ID, or 0.005" ID.

3.13.5.3. Mixing Tee: small internal volume PEEK, e.g. VICI JR-9000-0665.

3.14. Nylon filter 0.45 µm

4. REAGENTS AND MATERIALS

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade. Solvents shall be of HPLC or better quality and only double-distilled water or water of at least grade 2 as defined in EN ISO 3696 shall be used.

4.1. Double distilled or deionised water

4.2. Methanol

4.3. Acetonitrile

4.4. Potassium chloride (KCl)

4.5. Sodium chloride (NaCl)

4.6. Disodium hydrogenphosphate dodecahydrate (Na_2HPO_4*12 H_2O)

4.7. Disodium tetraborate decahydrate ($Na_2B_4O_7$ *10H_2O)

4.8. Sodium carbonate (Na_2CO_3)

4.9. Boric acid (H_3BO_3)

4.10. Potassium sulphate (K_2SO_4)

4.11. N-Acetyl-L-Cystein (NAC)

4.12. o-Phtalaldehyde (OPA)

4.13. β-Mercaptoethanol (BME)

4.14. Formic Acid (98-100%)

4.15. Phosphate buffered saline (PBS) concentrate

Dissolve the following in 1800 ml of water (4.1):
4 g KCl (4.4)
160 g NaCl (4.5)
72 g Na_2HPO_4*12 H_2O (4.6)

Adjust to pH 7.4 with 10 M HCl and make up to 2000 mL.

4.16. PBS Ready to use

Dilute 100 ml of PBS concentrate (4.15) to 1000 ml with water.

13.2. or

13.3. Phosphate buffered saline (PBS) tablet: e.g. Sigma P4417

One tablet dissolved in 200 mL of water (4.1) yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C.

4.17. Diluent

Mix 50 parts per volume methanol (4.2) with 50 parts per volume water (4.1).

4.18. Extraction solvent:

13.4. Mix 50 parts per volume methanol (4.2) with 50 parts per volume of PBS (4.16).

4.19. Reaction buffer:

4.19.1. Post-column derivatisation: (0.006 mol/L OPA, 0.006 mol/L NAC, 0.384 mol/L sodium carbonate, 0.216 mol/L boric acid and 0.108 mol/L potassium sulphate).

- Dissolve 40.7 g sodium carbonate (4.8), 13.4 g boric acid (4.9) and 18.8 g potassium sulphate (4.10) per 1.0 L of water (4.1).
- Stir for 10 min.
- Add 800 mg of OPA (4.12) per 1.0 L of the above solution.
- Add 1 g of NAC (4.11) per 1.0 L of the above solution.
- Stir for 10 min.

- Sonicate for 15 min.
- Stir for 10 min.
- Sonicate again for 15 min.
- Filter the solution through a 0.45 µm nylon filter (3.14).
- **Proper dissolution of the OPA is very important!**
- The reaction buffer should not be changed within a sequence of HPLC runs.
- Prepare fresh for every sequence of HPLC runs.

4.19.2. Pre-column derivatisation: (0.1 mol/L OPA, 0.24 mol/L BME, 0.08mol/L disodium tetraborate, 16.7% methanol (w,w,w,v))

- Dissolve 40 mg OPA (4.12) in 1.0 ml methanol (4.2)
- Mix until completely dissolved
- Add 5.0 mL of a 0.1 mol/L solution of disodium tetraborate decahydrate (3.8g/100mL; 4.7)
- Mix thoroughly
- Add 50 µL of BME (4.13)
- Mix thoroughly

Alternatively:

- **Phthaldialdehyde Reagent** (Sigma-Aldrich P0532)

4.20. FB_1 & FB_2 stock solution

Biopure Mycotoxin Mix 3 (Fumonisins)
A solution of FB_1 & FB_2 in acetonitrile/water (1/1), concentrations see label or certificate.

4.21. FB1 & FB2 diluted stock solution for calibration

- Add 160 µL of the FB_1 & FB_2 stock solution (4.20) to a 2 mL volumetric flask (3.10).

- Make up to mark (2.0 mL) with diluent (50% methanol, 4.17).

- This will result in 2.0 mL of a 12.5 fold dilution of 4.20 in methanol/acetonitrile/water (46/4/50, v/v/v), respectively.

- **Prepare this diluted stock solution twice!**

4.22. Calibration solutions

From each of the two diluted stock solutions (4.21) prepare five levels of calibration solutions by adding the volumes of diluted stock solution listed in the following table to a volumetric flask (3.10) of the indicated volume and make up to the mark with diluent (4.17).

Calculate the concentrations of FB_1 & FB_2 for the different calibration levels by dividing the stated concentrations of the stock solution (4.20) by the final dilution stated above. Should you observe saturation of the detector signal at the highest calibration level dilute 250 µL of diluted stock solution into 2.0 mL for a final dilution of 100.

Calibrant	Diluted stock solution (4.21) [µL]	Volumetric flask (3.10) [mL]	Final dilution of stock solution (4.20)
1	50	20.0	5000
2	125	10.0	1000
3	125	5.0	500
4	500	2.0	50
5	1000	2.0	25

4.23. Immunoaffinity columns

13.5. The immunoaffinity columns must contain a stationary phase with immobilized monoclonal antibodies specific to, at least, Fumonisin B_1 and B_2. To be suitable for this method they must meet the requirements stated below:

An aliquot of an extract of a blank compound animal feed material is spiked with FB_1 & FB_2 at either 920 (high) or 110 (low) ng/mL for the sum of both. Then dilute 5.0 mL of that spiked extract to a total volume of 50.0 mL (see Sec 6.1).

Following the procedures described in Sec 6.2 and 6.3 this will result in expected concentrations in the injection solutions of either 460 or 55 ng/mL for the sum of FB_1 & FB_2.

After measuring (Sec. 7) these solutions the observed concentrations of FB_1 & FB_2 can be calculated with Equ. I and II of Sec. 8. Dividing the sum of the observed concentrations of FB_1 & FB_2 by the expected concentrations will result in the yield of the immunoaffinity columns.

These yields must be 99%±18% (U, k=2) at the high level and 118%±18% (U, k=2) at the low level.

The above column test should be performed for each level on at least three randomly selected columns of every new lot of immunoaffinity columns which will be used. Should the tested lot not meet the above requirements either a new lot which does should be obtained or the conditions described in Sec. 6.2 need to be adjusted such that the requirements are met (the user instructions supplied with the columns are a good starting point).

!!!Any changes to the clean-up procedures will necessitate a revalidation of the clean-up and all subsequent steps (chromatography)!!!

4.24. Test materials

Homogenized, ready-to-be-extracted, and coded; to be prepared once each.

4.25. Spiking material

Homogenized, ready-to-be-extracted material for recovery determination; to be prepared once each.

4.26. Spiking solutions

FB_1 & FB_2 in a solution of acetonitrile/ water (1/1, v/v).

5. SPIKING PROCEDURE

To 20.0 g of spiking material (4.25) add a defined volume of a spiking solution (4.26). Let stand for 30 min before proceeding with the sample preparation (Sec. 6). Find details in the Spiking Protocol included with the documentation.

6. SAMPLE PREPARATION

6.1. Extraction of FB_1 & FB_2

- Weigh 20.0 g, to the nearest 0.1 g, of the test sample into a large enough container with lid, e.g. 250 mL flask (3.1).

- Add 200.0 mL of extraction solvent (4.18), cap, and shake vigorously by hand, so that the material disperses evenly.

- Put on a shaker (3.2) for 120 min. Choose speed such that the material is mixed well without collecting in the top of the flask.

- Allow the extracted sample to settle after shaking.

- Of the supernatant take 5.0 mL and dilute with PBS (4.16) to a total volume of 50.0 mL and mix.

- Prepare a filter funnel (3.7) with a glass micro fibre filter (3.6).

- Filter the diluted supernatant of the extracted sample into a new flask (3.1).

- The diluted filtered extract may be stored at 4 to 10 ºC overnight.

- In case of a **highly contaminated material above 10000 µg/kg** (see Sec. 8) take 10.0 mL of the stored filtered diluted extract and dilute again with PBS (4.16) to a total volume of 50.0 mL and mix.

6.2. Clean up

- Take one immunoaffinity column (IAC, 4.23) per extract.

- Attach a reservoir (3.9), **do not** empty storage solution from column.

- To the reservoir add 25.0 mL of the filtered diluted extract (6.1).

- Open the column outlet.

- Allow everything to pass slowly through the column. Flow rate should be about one drop per second.

- After the extract has passed completely, wash the IAC with 10 mL of PBS (4.16).

- Pass air through the IAC (e.g., using a properly fitted large syringe) in order to expel excess PBS.

- Place a 5 mL volumetric flask (3.10) or a 5 mL graduated cylinder (3.3) underneath the IAC and add 5 x 500 µL of methanol (4.2) to the IAC (add next aliquot only after previous has completely passed).

- Collect the eluate in the volumetric flask (3.10) or graduated cylinder (3.3).

- Add 2.0 mL of water (4.1) after all of the methanol (4.2) has passed through the column.

- Continue to collect the eluate in the same volumetric flask or graduated cylinder.

- Carefully pass air through the column in order to collect most of the applied water (4.1).

6.3. Test solution

- **For pre-column derivatisation**: Make up the content of the volumetric flask or graduated cylinder to the 5 mL mark with water (4.1).

- **For post-column derivatisation**: add 5uL of formic acid (4.14) and make up the content of the volumetric flask or graduated cylinder to the 5 mL mark with water (4.1).

- Mix the content of the volumetric flask or graduated cylinder and transfer an aliquot to an autosampler vial (3.8).

- This test solution may be stored at 4 to 10 ºC for up to two days.

7. MEASUREMENTS:

7.1. HPLC operating conditions

Below we provide recommendations for the operating conditions. More likely than not you will have to adjust injection volumes, the percentage of organic modifier in isocratic or gradient mode, the flow rate, and/or the column temperature to obtain appropriate resolution and retention (3.13.3) for your equipment.

DO NOT USE A MOBILE PHASE ADDITIVE OTHER THAN FORMIC ACID IN THE INDICATED CONCENTRATIONS!

To facilitate any necessary optimization work an additional container with a blank material which does not contain detectable amounts of FB_1 & FB_2 and a spare immunoaffinity column is included. Prepared acc. to Sec. 6 the resulting injection solution may be used for a few days. Compare the blank injection solution to an aliquot which has been spiked with a standard to identify possibly co-eluting matrix related peaks. Try to obtain sufficient peak resolution. That is especially relevant for pre-column derivatisation!

7.1.1. Pre-column derivatisation

Using the equipment outlined in 3.13, the following conditions have shown to produce satisfying results:

Auto sampler injector program:

- Aspire 20 µL pre-column reaction buffer (4.19.2)
- Aspire 40 µL test solution (6.3)
- Aspire 20 µL pre-column reaction buffer (4.19.2)
- Mix 20 times
- Inject all

The above can be done manually (adjusting the total volume while maintaining the relative volumes if necessary) if it is ascertained that the solution is injected **within 3**

min after mixing. It is also important that the **time period** between mixing and injecting is the **same** for all **test** and **calibration** solutions.

Injection volume: 80 µL

Column temperature: 40°C

Flow : 1.0 mL/ min

Fluorescence detector: Excitation λ: 335 nm; Emission λ: 440 nm **(it should be checked if these are local maxima for the fluorescence detector in use!)**

Mobile phase: A: 0.5% formic acid (4.14) in water (4.1)
B: 0.5% formic acid (4.14) in methanol (4.2)
(do not use any other additive than formic acid!)

Gradient settings (HPLC dwell volume 0.8 mL):

Time [min]	B [%]
0	69.5
14	79
14.01	100
17.01	100
17.02	69.5
20	69.5

Instruments with different dwell volume will need adjustment of the gradient to achieve the same separation as shown in Appendix A. The aim should be an apparent capacity factor at elution for FB_1 of $\boldsymbol{k > 3}$.

7.1.2. Post-column derivatisation

Instructions for self-assembled system:

The flow path to the chromatographic column (3.13.3) is unchanged from normal operation. The outlet of the column is connected to one of the outside ports of a mixing Tee (3.13.5.3). The tubing from column to mixing Tee should be as short as possible.

The other outside port of the mixing Tee is connected to the outlet of a pump (3.13.5.1) delivering the reagent flow. This connection should be made of a long piece of 0.005" ID PEEK tubing (3.13.5.2) so that a sufficient back pressure is created for the reagent pump to work properly. It is of utmost importance that the reagent flow is delivered pulsation-free. A slight pulsation can be minimized be introducing a large damping volume between the pump and the back pressure creating PEEK tubing. Large ID PEEK tubing can serve this purpose.

The remaining centre port of the mixing Tee is connected through a reagent loop to the fluorescence detector. The length, and therefore the volume, of this reagent loop is a balance between retaining the resolution of the chromatographic column (short) and achieving complete reaction (long). The internal diameter is of lesser importance. If chosen too small excessive back pressure will be created. Satisfying results were achieved with a 2.5m length of 0.02" ID PEEK tubing.

Using the equipment outlined in 3.13, the following conditions have shown to produce satisfying results:

Injection volume: 50 μL

Column temperature: 45°C

Flow : 1.2 mL/ min (mobile phase); 0.45 mL/min (post-column reagent (4.19.1))

Fluorescence detector: Excitation λ: 335 nm; Emission λ: 440 nm **(it should be checked if these are local maxima for the fluorescence detector in use!)**

Mobile phase: A: 0.1% formic acid (4.14) in water (4.1)
B: 0.1% formic acid (4.14) in acetonitrile (4.3)
(do not use any other additive than formic acid!)

Gradient settings:

Time [min]	B [%]
0	34
13	34
13.01	95
16	95
16.01	34
19	34

This separation is isocratic but to avoid accumulation of matrix compo–nents a step up to 95% B is included. The percentage of organic modifier should be adjusted such that the capacity factor for FB1 will be $k > 2$.

7.2. Determination of fumonisins in test solutions

Inject aliquots of the test solutions (6.3) into the chromatograph using the same conditions as used for the calibration solutions (4.22).

7.3. Batch (Sequence) composition

Each of the included test materials (4.24) and spiking materials (4.25) are to be prepared once. For each batch of runs all the calibration solutions (4.22) are to be run at the beginning and at the end of the batch. At the beginning of each batch run the five levels of calibrants prepared from the first diluted stock solution (4.21) from lowest to highest concentration followed by the five levels from the second diluted stock solution from highest to lowest. Then run the test solutions (6.3) once. Repeat the runs of the test solutions in reversed order for a second injection. At the end of the batch repeat the calibration solutions in reversed order from the beginning. Therefore, a batch of runs would, for example, look like Table 1 below.

7.4. Calibration

Plot the signals (peak area or height) of all the measured calibration solutions against the corresponding concentrations for FB_1 and, separately, for FB_2. Do not use means of the multiple injections! With linear regression estimate slope and intercept of each of the two

calibration functions (FB_1 & FB_2). Check for significance of the intercept and for linearity (use e.g. a residuals vs. fitted-values plot).

7.5. Peak identification

Identify the Fumonisin B_1 & B_2 peaks in the test solution by comparing the retention times with those of the calibration solutions. The signal (peak area or height) of FB_1 or FB_2 in the test solution must fall within the calibration range. If the FB_1 and/or FB_2 signal in the test solution exceeds the signals of the highest calibration solution the test solution shall be diluted with diluent (4.17) to bring it within calibration range, and be reanalysed. The dilution factor must be incorporated into all subsequent calculations.

Run	Identity	Run	Identity	Run	Identity
1	Cal A level 1	12	Material B	23	Cal B level 1
2	Cal A level 2	13	Material C	24	Cal B level 2
3	Cal A level 3	14	Material D	25	Cal B level 3
4	Cal A level 4	15	Material E	26	Cal B level 4
5	Cal A level 5	16	Material F	27	Cal B level 5
6	Cal B level 5	17	Material F	28	Cal A level 5
7	Cal B level 4	18	Material E	29	Cal A level 4
8	Cal B level 3	19	Material D	30	Cal A level 3
9	Cal B level 2	20	Material C	31	Cal A level 2
10	Cal B level 1	21	Material B	32	Cal A level 1
11	Material A	22	Material A		

Table 1: Example for the order of runs in a batch of runs

8. DETERMINATION OF CONCENTRATIONS

Using the estimated slopes and intercepts (if significant, otherwise use zero) from linear regression (7.4) calculate the concentrations of FB_1 ($c_{T(FB1)}$) and FB_2 ($c_{T(FB2)}$) in the test solutions (6.3) from the mean signal of the two injections as follows:

$$c_{T(FB1)} = \frac{\overline{\text{signal}}_{FB1} - \text{intercept}_{FB1}}{\text{slope}_{FB1}} \text{[ng/mL]} \qquad \text{(I)}$$

$$c_{T(FB2)} = \frac{\overline{\text{signal}}_{FB2} - \text{intercept}_{FB2}}{\text{slope}_{FB2}} \text{ [ng/mL]} \quad \text{(II)}$$

If the test solution was diluted because of a signal above the calibration range (7.5) multiply the calculated concentrations of FB_1 ($c_{T(FB1)}$) and FB_2 ($c_{T(FB2)}$) with the dilution factor.

To calculate the mass fractions (c_{SMP}) of the analytes in the original materials use the following equation:

$$c_{SMP} = \frac{c_T \times V_5 \times V_3 \times V_1}{V_4 \times V_2 \times m_{SMP}} \text{ [ng/g or µg/kg]} \quad \text{(III)}$$

with

c_T = calculated concentration of either FB_1 (I) or FB_2 (II), possibly corrected for dilution.

m_{SMP} = weight of the test material used for extraction (20.0 g).

V_1 = total volume of the extraction solvent (200.0 mL).

V_2 = volume of the aliquot of the filtered raw extract used for dilution (5.0 mL).

V_3 = total volume of the diluted filtered raw extract (50.0 mL).

V_4 = volume of the aliquot of the diluted filtered raw extract applied to the IAC (25.0 mL).

V_5 = total volume of the test solution (5.0 mL).

If the weight of the test material and the volumes described herein before are kept the above equation (III) can be simplified to:

$$c_{SMP} = c_T \times 20 \text{ [µg/kg]} \quad \text{(IV)}$$

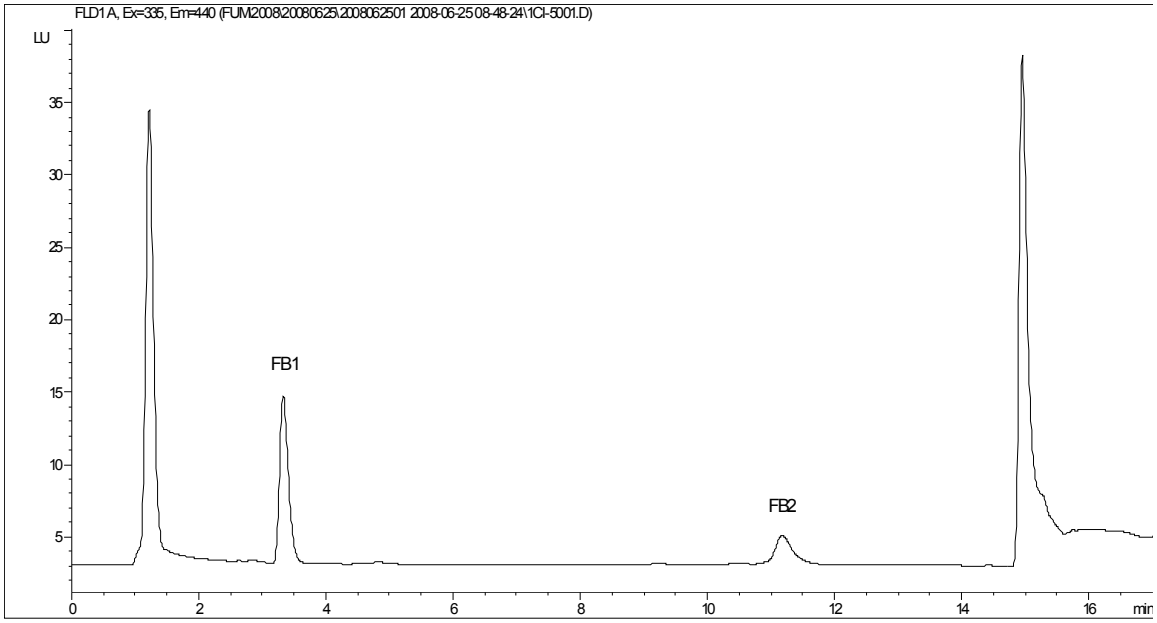
Should the result of equation IV be larger than **10000 µg/kg** or if it is known before hand that the contamination level might exceed that value clean-up the respective diluted filtered extract (see Sec. 6.1) using an additional dilution (additional dilution factor 50/10 = 5). The simplified equation will then be:

$$c_{SMP} = c_T \times 20 \times 5 = c_T \times 100 \text{ [µg/kg] (V)}$$

Carry out the above calculations for FB_1 and FB_2.

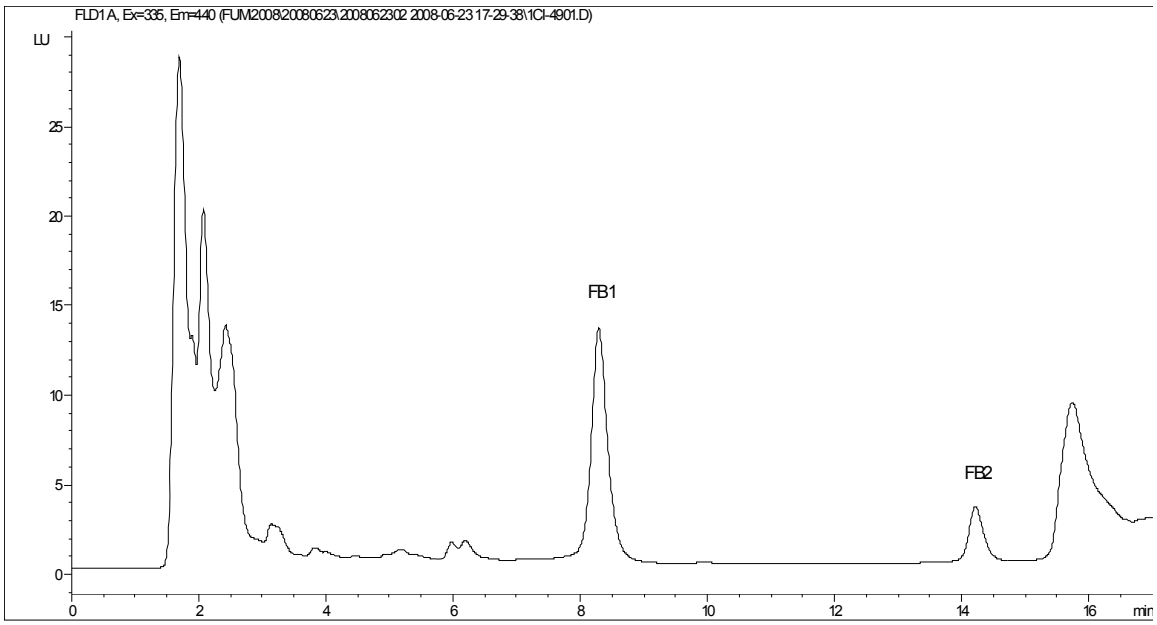
9. Appendix A:

9.1. Example chromatogram with post-column derivatisation and the conditions described herein before.

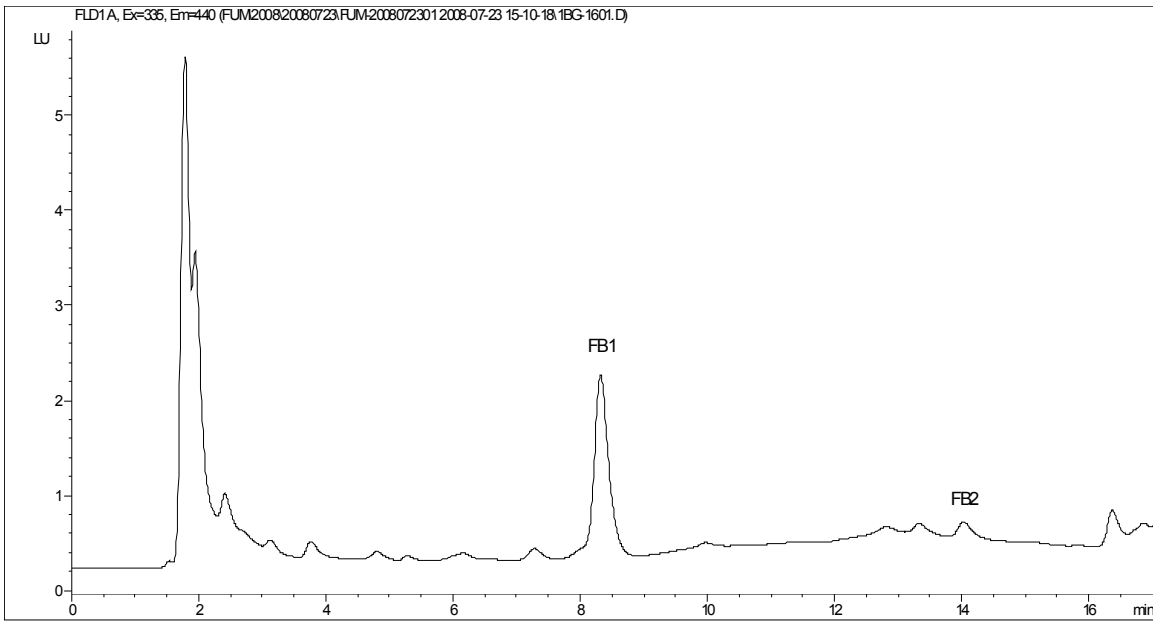


Animal feed blank material spiked at a level of ca. 5 mg/kg for the sum of FB_1 & FB_2. IAC eluate was dried down and reconstituted with 0.5 mL mobile phase.

9.2. Example chromatograms with pre-column derivatisation and the conditions described herein before.



Animal feed blank material spiked at a level of ca. 5 mg/kg for the sum of FB_1 & FB_2. IAC eluate was dried down and reconstituted with 0.5 mL mobile phase.



Animal feed material naturally contaminated at a level of ca. 6 mg/kg for the sum of FB_1 & FB_2. IAC eluate was not dried down.

Cover letter:

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements
Food safety and quality

Geel, 26 September 2008
D08/FSQ/ABR/bk/D (2008) 24453

Dear participant,
I would like to welcome you to a collaborative study to validate a method for the determination of the sum of Fumonisin B_1 & B_2 in compound animal feed. With this letter you will have received the materials and documentation necessary to carry out the analyses.
In order to make this retrial a success it is of **utmost importance** that you read the included documentation carefully. The protocol has changed from previous versions you might have received (see Foreword).
Please fill out the material receipt form and send it back to us. The materials you received have to be refrigerated until analysis. The analysis should only be started once you are certain that all the needed equipment (Protocol Sec. 3) and reagents (Protocol Sec. 4) are available. Also use the included blank material and one spare IAC column to prepare a blank injection solution with which the gradient settings can be optimized for your HPLC (Protocol Sec. 7). You have eight weeks until early December for reporting which should be sufficient time.
Prepare each of the ten materials once and determine their fumonisin concentrations as described in the protocol. Then fill out the report form and, preferably, email it to us. Keep all the data concerning this trial at hand in case we need to come back to you for clarification.
Once you have carried out all the analyses, please, also fill out the questionnaire and email it back to us. Receiving the electronic versions of those forms will facilitate our work very much.

Should you have comments or questions, please, do not hesitate to contact us at jrc-irmm-crl-mycotox@ec.europa.eu
The deadline for reporting is 01 Dec 2008.

Thanking you for your participation we remain
With kind regards

Andreas Breidbach

Contact:
Andreas Breidbach, Telephone:+32-(0)14-571 205, andreas.breidbach@ec.europa.eu

SPIKING PROTOCOL

This box contains two containers labelled "Spike A" and "Spike B". These are materials to be used for recovery determinations.

There are also two amber ampoules labelled "Spike A" and "Spike B". These are solutions of FB1 & FB2 in acetonitrile/ water (50/50, v/v).

To determine the recovery of the method proceed as follows (the references in parentheses refer to the method protocol):

- Weigh 20.0 g, to the nearest 0.1 g, of the test sample into a large enough flask with lid, f.i. 250 mL flask (3.1).
- Carefully tap the amber ampoules on the bench so as to collect all liquid at the bottom of the ampoule.
- Wrap ampoule in some heavy tissue and wearing sturdy gloves carefully break off the top of the ampoule at the mark (glass surface pre-scored).
- Add the volume of spiking solution indicated in the table below to the test sample in the flask.

Material	Solution	Volume
Spike A	Spike A	600 µL
Spike B	Spike B	500 µL

- Apply the solution such that it is distributed over a wide area of the sample material. Do not apply to the walls of the flask. After one minute gently shake the flask to further distribute the application area (only side-to-side motion, not up-down).
- Let stand for 30 min at room temperature to allow the acetonitrile to evaporate.
- Shake to distribute the contaminated material evenly and tap flask on bench to collect all material at the bottom of the flask.
- Proceed with the addition of 200.0 mL of extraction solvent (4.18) in section **6.1 Extraction of FB1 & FB2** in the method protocol.

Fumonisin Method Validation Study 2008
Questionnaire

Please **read** this questionnaire **before** you start the analysis and **complete** it **after** you have carried out the analysis.

Analyst:	
Laboratory:	

1 Did your laboratory already perform Fumonisin analysis prior to this study?

○ Yes (go to 2) ○ No (go to 3)

2 If you did, please, briefly describe the methodology used in the fields below.

Extraction solvent:		Separation:	
Clean-up:		Detection:	

For how long have you been performing Fumonisin analysis?

○ < 3 months ○ 3 - 12 months ○ > 12 months

3 If you did not, describe which parts of the proposed method were unfamiliar to you in the field below?

4 Was the description of the method adequate?

○ Yes (go to 6) ○ No (go to 5)

5 What could be improved about the method description?

6 Did you at any step deviate from the method protocol sections 4, 5, or 6?

○ Yes (go to 7) ○ No (go to 8)

7 If you did, please state where and why in the field below.

8 Approximately, how long did the clean-up step (6.2. of the method protocol) for an individual sample take on average?

○ < 20 min ○ 20 - 40 min ○ > 40 min

9 Please describe the HPLC instrument used in the fields below.

Pump:		Detector:	
ALS:		Column:	

10 Did you use pre- or post-column derivatization? Please describe set-up briefly in the field below.

○ Pre ○ Post

11 Did you deviate from method protocol section 7 (Measurements)?

○ Yes (go to 12) ○ No (go to 13)

12 If you did, please state where and why in the field below.

13 Did you use peak area or peak height for evaluation?

○ Peak area ○ Peak height

Thank you for completing this questionnaire!

Once you filled-in this form **save it**! To facilitate our work we ask you to submit the electronic version by using the "Submit by Email" button. Should that not work use the "Print Form" button and fax or mail it to:

JRC-IRMM
A. Breidbach
Retieseweg 111
B-2440 Geel
Belgium
Fax: ++32 14 571 783.

Submit by Email Print Form

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements
Food safety and quality

MATERIALS RECEIPT FORM

Name and Institution:

NOTE: UPON RECEIPT STORE ALL MATERIALS IN A REFRIGERATOR AT 4 °C!

Please ensure that the items listed below have been received undamaged, and then check the relevant statement:

Date of the receipt	
Items are missing/damaged. I require a replacement.	○ Yes ○ No
Specify here what you want replaced:	

Contents of parcel

a) Six test material containers identified by a code

b) Two test material containers identified as “Spike A”

c) Two test material containers identified as “Spike B”

d) Two amber ampoules identified as “Spike solution A”

e) Two amber ampoules identified as “Spike solution B”

f) One test material container identified as “Animal feed blank”

g) 14 immunoaffinity columns

h) Vial with Fumonisin Mix reference material (if it was requested)

i) Documentation: Method protocol, Spiking protocol, Cover letter, Copy of Certificate of Fumonisin Mix reference material (if it was requested), Results form, Questionnaire form, this form

Please fax or email the completed form to:

Dr. Andreas Breidbach
European Commission - Joint Research Centre
Institute for Reference Materials and Measurements
B-2440 Geel, Belgium
Fax No: 0032-14-571 783

Submit by Email Print Form

Fumonisin Method Validation Study 2008
Results

Analyst:	
Laboratory:	

Instructions:
Please enter in the provided fields sample codes and corresponding concentrations for FB_1 & FB_2 as calculated using Eq. III, IV, or V of section 8 of the method protocol. **Enter the concentrations in µg/kg rounded to full unit, e.g. 1181.4 as 1181!**

Once you filled-in the form **save it**! To facilitate our work we ask you to use the "Submit by Email" button to email to us the electronic version. Should this not work use the "Print Form" button to print it and fax or mail it to: JRC-IRMM, A. Breidbach, Retieseweg 111, B-2440 Geel, Belgium; Fax: ++32 14 571 783

Sample code	Concentration FB1		Concentration FB2	
Spike A 1		µg/kg		µg/kg
Spike A 2		µg/kg		µg/kg
Spike B 1		µg/kg		µg/kg
Spike B 2		µg/kg		µg/kg
		µg/kg		µg/kg
		µg/kg		µg/kg
		µg/kg		µg/kg
		µg/kg		µg/kg
		µg/kg		µg/kg
		µg/kg		µg/kg

Submit by Email

Print Form

European Commission

EUR 23941 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: REPORT OF THE FOLLOW-UP COLLABORATIVE STUDY
Author(s): A. Breicbach, K.Bouten, K. Kroeger, J. Stroka
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EUR – Scientific and Technical Research series – ISSN 1018-5593
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DOI 10.2787/12806

Abstract
A collaborative study to validate a method to determine the sum of Fumonisin B_1 (FB_1) and B_2 (FB_2) in compound animal feed and maize by immunoaffinity column clean-up and High Performance Liquid Chromatography with Fluorometric Detection (HPLC-FLD) was conducted by the Joint Research Centre – Institute for Reference Materials and Measurements with the participation of 16 laboratories of 12 Member States of the European Union.

The method consisted of extraction of the Fumonisins from the sample with 50 % methanol in phosphate-buffered saline, clean-up with immunoaffinity columns, and detection of the o-phtaldialdehyde derivatives after either pre- or post-column derivatisation with HPLC-FLD.

Five materials, consisting of compound animal feed and maize, were tested. Three of the materials were naturally contaminated, and two were naturally contaminated and additionally spiked. The contamination levels ranged from 3 to 16 mg/kg for the sum mass fraction of FB_1 and FB_2.

Four laboratories had to be excluded from the evaluation because of non-compliance. For the remaining 12 laboratories repeatability and reproducibility were computed using robust statistical methods. Relative repeatability and reproducibility standard deviations of 2.0 to 5.3 %, and 15 to 20%, respectively, were found. Horwitz ratios ranged from 1.2 to 1.9 and were all acceptable.

We deem the method as fit for the purpose of enforcing recommended maximum levels of the sum of Fumonisin B_1 and B_2 in animal compound feed, and maize intended for animal feed, and have submitted it to the European Committee for Standardisation (CEN)/ Technical Committee (TC) 327 Animal feeding stuffs.

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